

THESIS

IMPACT OF TIMING OF MILK INTAKE ON NITROGEN BALANCE IN  
HYPOCALORIC EXERCISING OLDER INDIVIDUALS

Submitted by

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## ABSTRACT

### IMPACT OF TIMING OF PROTEIN INTAKE ON NITROGEN BALANCE IN HYPOCALORIC EXERCISING OLDER INDIVIDUALS

We have previously shown that in older adults, consumption of protein immediately after aerobic exercise, rather than earlier in the day, enhances nitrogen balance when energy balance is maintained. Since some older individuals consume lower calorie diets, it is important to know if these benefits also occur during hypocaloric feeding. The purpose of the study was to investigate if consumption of protein immediately after aerobic exercise rather than earlier in the day can improve nitrogen balance in older individuals consuming a hypocaloric diet. In a randomized crossover design, healthy sedentary male ( $n=2$ ; age= $67.0 \pm 1.0$  years; BMI= $27.4 \pm 0.3$  kg/m<sup>2</sup>) and female ( $n=8$ ; age= $63.0 \pm 1.8$  years; BMI= $22.3 \pm 0.6$  kg/m<sup>2</sup>) subjects completed two separate 3-day exercise and nutrition interventions. Exercise (60 minutes of stationary cycling at 55% of  $\text{VO}_{2\text{max}}$ ) was performed daily. Diets were hypocaloric (-15% daily intake), with a protein+carbohydrate (PRO+CHO) or carbohydrate only (CHO) drink consumed in the morning and the opposite drink consumed after exercise. Both diets (15% protein, 30% fat, and 55% carbohydrate) were isonitrogenous and isocaloric with only the timing of the drinks differing. A 24-hour stay in a metabolic chamber confirmed negative energy balance, while 24-hour urine collections determined nitrogen balance. The 3-day mean

nitrogen balance was not significantly greater in the PRO+CHO trial ( $0.097 \pm 0.526$ g N) trial than the CHO trial ( $-0.070 \pm 0.520$ g N) ( $p=0.280$ ). Thus, older individuals in negative energy balance do not maintain a significantly more positive nitrogen balance by consuming protein after aerobic exercise as opposed to earlier in the day. These results differ from our previous work and indicate that energy balance is an important determinant of the anabolic effect of protein feeding.

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## CHAPTER 1

### INTRODUCTION

Sarcopenia is the gradual reduction of skeletal muscle mass and function associated with aging. While there is not an accepted clinical definition of sarcopenia, the most commonly used definition is based on a skeletal muscle mass index obtained by dividing appendicular skeletal muscle by body height squared ( $ASM/ht^2$ ) (Baumgartner, Koehler et al. 1998). Individuals with an  $ASM/ht^2$  ratio between one and two standard deviations of the gender-specific young control are categorized as having class I sarcopenia and individuals exceeding two standard deviations are considered to have class II sarcopenia (Janssen, Heymsfield et al. 2002). The prevalence of class I sarcopenia is approximately 52%, whereas the prevalence of class II is about 8% in individuals aged 60 and older. By the age of 70, the cross-sectional area of skeletal muscle can be reduced by up to 25-30% and muscle strength may be reduced by 30-40% (Porter, Vandervoort et al. 1995), with the loss of strength continuing to decrease at a rate of 1-2% per year (Skelton, Greig et al. 1994). The age-related loss of muscle mass and strength results in a decline in functional independence, physical disability, and mobility impairment (Janssen, Heymsfield et al. 2002). Therefore, the sarcopenic older individual has greater difficulty doing basic activities of daily living, which increases their risk of falls and fractures. Sarcopenia also contributes to the pathogenesis of frailty, which is associated with increased hospitalizations, morbidity and mortality (Rolland, Czerwinski et al. 2008). Although multiple lifestyle behaviors and mechanisms contribute to the development of sarcopenia, the loss of lean body mass is the result of differences in rates of protein breakdown and protein synthesis.

The nitrogen balance method is commonly used to measure whole body protein balance. Protein is the major nitrogen-containing substance within the body, and a gain or loss of nitrogen can be considered as synonymous with a gain or loss of protein (Calloway and Spector 1954). When nitrogen intake exceeds the amount of nitrogen excreted, an individual is in positive nitrogen balance and when protein intake is less than the amount of nitrogen excreted negative nitrogen balance results. Nitrogen balance is closely related to energy balance such that nitrogen balance is better maintained when caloric intake is adequate, as a negative nitrogen balance results when energy intake is reduced (Todd, Butterfield et al. 1984). During caloric restriction, protein breakdown increases the availability of amino acids that are oxidized as energy and thus limits the availability of amino acids for protein synthesis.

Nutritional interventions that aim to maintain or increase lean body mass have primarily focused on increasing protein intake above the RDA of 0.8 g/kg bw. The current RDA of 0.8 g/kg bw may not be adequate for older individuals as Campbell et al. have demonstrated that when older individuals in energy balance consumed the RDA for protein, negative nitrogen balance and loss of mid-thigh muscle area results (Campbell, Trappe et al. 2001). However, in a recent study by Campbell et al., older individuals in energy balance maintained nitrogen balance with the RDA (Campbell, Johnson et al. 2008). The recent study by Campbell et al. eliminated weaknesses of previous studies by including only healthy men and women subjects, using young subjects as a control group, and allowing adequate study time periods (Campbell, Johnson et al. 2008). Rather than increasing total protein intake, another approach to maximize net protein accretion in older individuals is to consume 25-30 grams of high quality protein per meal (Paddon-Jones and Rasmussen 2009). Although increasing the absolute amount of protein is debatable, nutritional interventions represent a practical option for older individuals to maintain lean body mass. In older individuals, exercise represents another strategy for maintaining lean



body mass. Resistance exercise can increase muscle cross sectional area and strength in older individuals (Frontera, Meredith et al. 1988). Additionally, recent studies have shown that aerobic exercise can also stimulate skeletal muscle protein synthesis (Wilkinson, Phillips et al. 2008) and myofiber size and function (Harber, Konopka et al. 2009) in older individuals. Moreover, even when caloric intake is inadequate, aerobic exercise can increase nitrogen balance (Todd, Butterfield et al. 1984). Because nutrition after exercise can take advantage of the anabolic period triggered by exercise (Miller, Olesen et al. 2005), combining nutrition with exercise may be the most effective strategy for preventing the loss of lean body mass in older individuals. However, there is conflicting evidence as to whether timing protein intake after exercise results in enhanced protein synthesis and thus lean body mass (Rasmussen, Tipton et al. 2000; Tipton, Rasmussen et al. 2001). In older individuals, protein consumption after aerobic exercise can increase whole body protein turnover (Murphy and Miller 2010) and nitrogen balance while in energy balance (Jordan, Melanson et al. 2010). Since many older individuals are in negative energy balance (McDowell, Briefel et al. 1994), the current study investigated whether protein consumption after aerobic exercise, rather than earlier in the day, can improve nitrogen balance in older individuals in negative energy balance.

**Statement of the Problem:**

The purpose of the study is to investigate the effect of protein intake immediately after moderate intensity aerobic exercise rather than earlier in the day on nitrogen balance in hypocaloric older individuals.

**Hypothesis:**

In older individuals in negative energy balance, timing protein consumption immediately after moderate aerobic exercise compared to earlier in the day will increase three-day nitrogen balance.

**Delimitations, Limitations, and Assumptions:**

The study was delimited to 10 male and female subjects between the ages of 55-75 years old. All subjects were recruited from the Fort Collins and Loveland area and were Caucasian.

There are limitations associated with the nitrogen balance technique, which includes potential overestimation of nitrogen balance (Hegsted 1976). However, subjects are exposed to similar study conditions during both three-day trials, so nitrogen balance would be overestimated similarly.

It was assumed that subjects fasted for 12-hours prior to their resting metabolic rate and blood draw. Additionally, it is assumed that subjects consumed only meals provided by study staff during the lead-in and inpatient periods.

## CHAPTER 2

### LITERATURE REVIEW

Aging is associated with a progressive loss of physical independence, which leads to a significant reduction in the quality of life of older individuals. A central contributor to frailty in older individuals is the loss of muscle mass, strength, and function. Although multiple lifestyle behaviors and mechanisms contribute to the development of sarcopenia, the loss of lean body mass is the result of differences in rates of protein breakdown and protein synthesis. Therefore, this review will first aim to review various topics associated with protein metabolism. Then, the condition of sarcopenia will be defined and discussed, with an emphasis on potential causes of sarcopenia including decreased anabolic signaling, oxidative stress, and various lifestyle behaviors that may be responsible for the loss of lean body mass. The next sections will then address the effect of nutrition, exercise, and energy balance on nitrogen balance and protein synthesis. Lastly, nutritional and exercise interventions for the management of sarcopenia will be covered.

#### **Section 1: Amino Acids/Protein**

Amino acids play central roles both as building blocks of proteins and as intermediates in metabolism. All amino acids have the same fundamental structure, which consists of a central carbon, an amino group, a carboxyl group and a unique side chain (R-group). The carboxyl of one amino acid reacts with the amino group of another to form a peptide bond between the amino acids. Amino acids joined together by peptide bonds form the primary structure of proteins. Ten of the twenty amino acids are nonessential amino acids and can be

synthesized endogenously. The remaining ten amino acids are essential amino acids that have to be obtained from the diet (Gropper, Smith et al. 2009).

The digestion of ingested proteins starts in the stomach where hydrochloric acid denatures quaternary, tertiary and secondary structure of proteins, which allows the peptide bonds to be exposed to pepsin. Within the stomach, pepsin hydrolyzes the peptide bonds between amino acids. Protein digestion continues into the small intestine where pancreatic proteases continue to hydrolyze peptide bonds. The combined action of gastric and pancreatic proteases digest proteins into oligopeptides and free amino acids. Intestinal brush border enzymes further digest the oligopeptides prior to active absorption of free amino acids (Walker 2000). Dipeptides and tripeptides are also transported across the brush border of intestinal enterocytes via PEPT1 transport protein where they are further hydrolyzed by cytoplasmic peptidases to free intracellular amino acids. Free amino acids not utilized by the enterocyte are transported through the basolateral membrane where they enter the portal vein and eventually reach systemic circulation (Gropper, Smith et al. 2009).

Free amino acids derived from dietary protein sources and from degradation of body proteins both contribute to a pool of free amino acids. This pool of free amino acids is maintained in order to supply tissues with a continuous supply of individual amino acids for the synthesis of new proteins and other nitrogen-containing compounds (Storey 2004). There is constant turnover of protein, as some protein is constantly being synthesized while other protein is being degraded. In basal situations (between meals), plasma amino acid concentrations remain relatively stable whereas the concentration increases following a meal (Dunford and Doyle 2008). Similarly, protein synthesis increase in the fed state when the concentration of free amino acids increases. Skeletal muscle, which acts as a major reservoir of amino acids, increases uptake of amino acids following ingestion of protein. During this time,

protein synthesis is typically greater than protein degradation (Gropper, Smith et al. 2009). When caloric intake is inadequate, the overall breakdown of proteins, especially in skeletal muscle, increases to provide amino acids that are essential for new protein synthesis and energy production (Lecker, Solomon et al. 1999). During a prolonged fast, amino acids derived from muscle protein are an important substrate for gluconeogenesis, which provides energy from non-carbohydrate sources (Ruderman 1975). Despite differences in protein intake and rate of degradation of tissue protein, the concentration of the amino acids in the free amino acid pool remain relatively constant.

Free amino acids are metabolized in response to various physiological states. Amino acids in excess of the amount needed to synthesize protein and other nitrogen containing compounds undergo deamination or transamination reactions. Transamination involves the transfer of an amine group from an amino acid to an  $\alpha$ -keto acid. Transamination reactions form nonessential amino acids from essential amino acids and other nonessential amino acids (Gropper, Smith et al. 2009). When amino acids are deaminated, the amino group is removed as ammonia and an  $\alpha$ -keto acid (carbon skeleton) is formed. The  $\alpha$ -keto acids can then be used as a substrate in energy producing pathways (gluconeogenesis) or to form fatty acids, which can then be utilized as energy or stored as fat (Lieberman, Marks et al. 2007). The ammonia produced through deamination is converted to urea and excreted in urine (Gropper, Smith et al. 2009). When energy intake is inadequate, amino acids can be used for energy producing reactions. When energy intake is in excess of the body's energy needs fatty acids can be generated from amino acids through the process of lipogenesis.

## **Section 2: Protein Metabolism**

### **Section 2.1: Protein Synthesis**

Protein synthesis involves the transcription of deoxyribonucleic acid (DNA) to ribonucleic acid (RNA), and the translation of RNA to proteins. Transcription begins when RNA polymerase binds to the promoter region of DNA. Once bound to the promoter, RNA polymerase unwinds the DNA sequence and synthesizes an exact copy of the DNA strand. The RNA transcript undergoes RNA processing, where a modified guanine cap is attached to the 5' end and the 3' end is polyadenylated. The intron sequences are then removed from the RNA transcript. The newly formed messenger RNA (mRNA) is then transported out of the nucleus into the cytoplasm, where translation occurs. Translation is initiated by the binding of the eukaryotic initiation factor (eIF), eIF-4E, to the cap structure at the 5' end of the mRNA. The eIF-4 complex is then recognized by a complex consisting of the 40S ribosomal subunit, the initiator transfer RNA (tRNA) and eIF-2. The 40S subunit scans the mRNA for the initiator codon AUG (methionine) and attaches at that point. Then the 60S ribosomal subunit binds to form the 80S ribosome, and translation is initiated. Following initiation of the peptide chain, elongation occurs in which amino acids are joined to create a polypeptide chain. During elongation, a specific eukaryotic elongation factor (eEF), eEF-1, recruits a tRNA with its associated amino acid to the ribosome. A peptide bond is then formed between the carboxyl groups of the last amino acid with the new amino acid. Subsequently, eEF-2 catalyzes the translocation of the ribosome three bases down the mRNA and the process of elongation is repeated. Elongation continues until one of the three stop codons is encountered by the ribosome, at which point a eukaryotic releasing factor (eRF) cleaves the newly formed peptide (Latchman 2005). Following the termination of translation, post-translational modifications occur that affect the final structure and function of the protein. Post-translational modifications can involve the removal of one or

more amino acids from the peptide chain, addition of a side group, or the modification of a side group (Whitford 2005). The multi-step process of protein synthesis results in the conversion of the genetic information within DNA into a protein.

### Section 2.2: Protein Breakdown

The majority of intracellular protein is degraded by the ubiquitin-proteasomal system (Lecker, Solomon et al. 1999). In the ubiquitin system, proteins that are to be degraded are ligated to ubiquitin in an ATP-requiring reaction. Before ubiquitin can be linked to a protein, it must first be activated by the enzyme E1. The newly activated ubiquitin is then transferred to the enzyme E2, which then transfers ubiquitin to the target protein in a reaction catalyzed by the E3 ubiquitin ligase. Ubiquitinated proteins are subject to either further rounds of ubiquitin addition, or ubiquitin removal by deubiquitinating enzymes and degradation by the 26S proteasome, which breaks down targeted substrates to short peptides but recycles the ubiquitin molecules. The 26S proteasome is a large multisubunit complex composed of a 20S proteasome, and a 19S regulatory particle. The 19S regulatory particle recognizes and binds ubiquitinated substrates, while the 20S proteasome degrades the target protein into amino acids (2009).

In addition to the ubiquitin-proteasomal pathway, protein degradation also occurs via a lysosome mediated pathway. The lysosomal pathway degrades proteins within the lysosome of the cell. Lysosomes contain several acidic proteases that are capable of hydrolyzing endocytosed proteins. Extracellular proteins brought into the cell by endocytosis, membrane bound proteins, and long-lived intracellular proteins are degraded in lysosomes in an ATP-independent pathway. Cytosolic proteins are degraded in lysosomes after being engulfed in autophagic vacuoles that fuse with lysosomes. The lysosomal pathway is responsible for the

enhanced protein degradation observed during caloric restricted conditions (Gropper, Smith et al. 2009).

In muscle, the calpain or calcium-activated protease pathway contributes to protein degradation.  $\mu$ -Calpain and m-calpain are the principle components of the calpain proteolytic pathway, and the activation of both proteases is modulated by intracellular calcium concentrations (Tavernarakis 2010). The calpains are localized in the Z disk of the sarcomere, where they initiate the first step of degradation of muscle proteins. Following the release of myofilaments from the myofibrils, the myofilament is then ligated to ubiquitin for further degradation via the ubiquitin-proteasomal pathway (Gropper, Smith et al. 2009). Cells contain multiple proteolytic systems and complex regulatory mechanisms for the breakdown of proteins.

### Section 2.3: Protein Turnover

Protein turnover encompasses the simultaneous synthesis and degradation of proteins. The turnover of protein is an essential process that replaces damaged proteins that arise in cells due to spontaneous denaturation, errors in protein synthesis, errors in posttranslational processing, improper folding of the protein or due to damage caused by free radicals (Lecker, Solomon et al. 1999). Additionally, protein turnover allows the body to rapidly alter the concentrations of specific proteins in response to various stimuli (Welle 1999). Lastly, turnover of protein maintains the free amino acid pool, which allows the demands of protein synthesis to be met during times of energy restriction. During times of energetic stress, protein degradation exceeds protein synthesis. The body replaces previously catabolized proteins during periods of caloric or nitrogen excess, thereby maintaining a protein balance (Liu and Barrett 2002). In weight stable individuals, whole body protein breakdown is approximately equal to whole body protein synthesis over a typical 24-hour period (Wagenmakers 1999). When energy and protein



(amino acid) intake are adequate, protein breakdown is reduced and net protein synthesis results.

The turnover rate of proteins can be determined using isotopically labeled tracers of amino acids. The rate of protein turnover varies between tissues and differences also exist between the fractional synthesis rates of individual proteins (Wagenmakers 1999). Protein turnover accounts for approximately 20% of an individuals' resting metabolic rate with most of the energy being devoted to protein synthesis rather than proteolysis (Welle 1999). In general, initial studies (Golden and Waterlow 1977; Lehmann, Johnston et al. 1989) have demonstrated reduced protein turnover rates with aging when values are expressed per kilogram body weight (Golden and Waterlow 1977; Lehmann, Johnston et al. 1989). However, these studies did not account for the changes in body composition of the older individuals. When protein turnover is expressed as protein turnover per kilogram of lean mass, there is no difference between the rates of protein turnover between young and older individuals in the postabsorptive state (Morais, Gougeon et al. 1997). Protein synthesis and degradation are closely regulated, and each is affected by various physiological conditions, such as fasting, feeding, exercise, and disease.

#### Section 2.4: Negative Energy Balance and Protein Turnover

Protein turnover is affected by changes in energy balance. In response to an acute (less than four days) hypocaloric feeding, whole body protein turnover is increased (Nair, Woolf et al. 1987; Knapik, Meredith et al. 1991). Whole body proteolysis increases while protein synthesis is decreased. The increased protein breakdown increases the availability of amino acids that can be used as energy during an energy deficit. The increase in amino acid oxidation, especially branched chain amino acids, also limits the availability of amino acids for protein synthesis. The activity of AMP-activated protein kinase (AMPK), regarded as the energy sensor of the cell,

increases in response to the decrease in available energy, which also inhibits protein synthesis through the mammalian target of rapamycin (mTOR) pathway (Miyazaki and Esser 2009). During acute caloric restriction, vital organs are better able to maintain their respective synthetic rates compared to skeletal muscle. An acute energy deprivation results in a 19% decrease in the synthetic rate of skeletal muscle, while the synthetic rate of the heart is maintained (Yuan, Sharma et al. 2008). During prolonged (8 weeks) caloric restriction, whole body protein turnover is decreased (Hoffer, Bistrian et al. 1984). However, there is conflicting research as to whether long term caloric restriction decreases protein turnover, as long term calorie restricted animals may have an increase in protein turnover (Lee, Klopp et al. 1999; Weindruch, Kayo et al. 2002). Protein turnover is energetically costly, therefore when energy intake is reduced, protein turnover will be down regulated.

#### Section 2.5: Skeletal Muscle Protein Turnover

Proteins in skeletal muscle, as in all tissues, undergo a continuous process of degradation and synthesis. Skeletal muscle is the major reservoir of the body's amino acids as it contains over 60% of free amino acids (Wagenmakers 1999) and contributes about 30% to the whole body protein turnover (Nair 1995), despite a relatively low fractional synthetic rate (FSR) of approximately 1.15%/day (Wagenmakers 1999). During starvation, muscle releases amino acids as a consequence of increased protein breakdown. The amino acids that are released from muscle can be used for synthesis of essential acute phase proteins that turnover more quickly than skeletal muscle (Lecker, Solomon et al. 1999) and as substrates in energy producing pathways (Mitch and Goldberg 1996). In the fed state, amino acids stimulate muscle protein synthesis, which results in a transition from net release (postabsorptive state) to net uptake of amino acids (postprandial state). The relative contribution of muscle to whole body protein turnover increases during ingestion of mixed meals and after resistance exercise. The muscle

contributes 35% to whole body protein turnover after consuming a mixed meal, and approximately 45% during ingestion of mixed meals after resistance exercise (Wagenmakers 1999). The anabolic effect of feeding and exercise allows skeletal muscle to replace proteins that were lost during times of energetic stress.

#### Section 2.6: Skeletal Muscle Protein Metabolism in the Elderly

Aging is associated with a progressive loss of muscle mass that occurs at a rate of 3 to 8% per decade after the age of 30 and accelerates with advancing age (Genaro Pde and Martini 2010). The contribution of muscle protein to whole body protein metabolism is significantly reduced in the elderly. Consequently, the contribution of non skeletal muscle protein, especially visceral tissue whose rates of protein turnover are known to be more rapid, is proportionally greater with aging (Morais, Chevalier et al. 2006). The decreased skeletal muscle mass could reduce the capacity of the elderly to respond to restricted energy and protein intakes or to stressful conditions that require mobilization of amino acids from the muscle for protein synthesis.

Essential amino acids are mainly responsible for the postprandial increase in muscle protein synthesis (Volpi, Kobayashi et al. 2003); however, the anabolic response of skeletal muscle proteins to essential amino acids is blunted in the elderly (Cuthbertson, Smith et al. 2005). In addition, skeletal muscle of older individuals is resistant to the anabolic action of insulin (Rasmussen, Fujita et al. 2006). Although the exact mechanism for the impaired response to both essential amino acids and insulin are not currently known, both appear to act through the mTOR pathway. In response to both essential amino acids and insulin, mTOR activation remains the same, but its downstream effector p70 ribosomal S6 kinase's (p70<sup>S6K</sup>) activation is impaired (Guillet, Prod'homme et al. 2004). The synthesis rate of mixed muscle protein and myosin heavy-chain is diminished in the elderly, which contributes to the decreased

contractile function of skeletal muscle in the elderly (Balagopal, Rooyackers et al. 1997). Aging is also associated with a decreased rate of mitochondrial protein synthesis in skeletal muscle, which could result in decreased ATP availability and an increased susceptibility to fatigue (Rooyackers, Adey et al. 1996).

### **Section 3: Sarcopenia**

Sarcopenia is the gradual reduction of skeletal muscle mass and function associated with aging. While there is no accepted clinical definition of sarcopenia, the most commonly used definition is based on a skeletal muscle mass index obtained by dividing appendicular skeletal muscle by body height squared ( $ASM/ht^2$ ) (Baumgartner, Koehler et al. 1998). Individuals with an  $ASM/ht^2$  ratio between one and two standard deviations of the gender-specific young control are categorized as having class I sarcopenia and individuals exceeding two standard deviations are considered to have class II sarcopenia (Janssen, Heymsfield et al. 2002). Depending on the definition used for sarcopenia, the prevalence in 60-70 year olds is 5-13%, while the prevalence ranges from 11- 50% in people 80 years or older (Morley 2008). In addition, the prevalence of class I sarcopenia is approximately 52%, whereas the prevalence of class II is about 8% in individuals aged 60 and older. The age-related loss of muscle mass and strength results in a decline in functional independence, physical disability, and mobility impairment (Janssen, Heymsfield et al. 2002). Therefore, the sarcopenic older individual has greater difficulty doing basic activities of daily living, and an increased risk of falls and fractures. Loss of muscle mass is associated with a low threshold of fatigue (Fleg and Lakatta 1988) and a reduced resting metabolic rate (Lammes and Akner 2006). Thus, individuals with sarcopenia have a reduced capacity for exercise and a decreased caloric requirement, which can further enhance the progression of sarcopenia. Sarcopenia also contributes to the pathogenesis of

frailty, which is associated with increased hospitalizations, morbidity and mortality (Rolland, Czerwinski et al. 2008).

The decrease in muscle mass that gives rise to sarcopenia involves both a decrease in muscle fiber size and number. By the age of 70, the cross-sectional area of skeletal muscle can be reduced by up to 25-30% and muscle strength may be reduced by 30-40% (Porter, Vandervoort et al. 1995), with the loss of strength continuing to decrease at a rate of 1-2% per year (Skelton, Greig et al. 1994). With aging, type II fibers atrophy more than type I fibers (Larsson, Sjodin et al. 1978) and the loss of lean body mass is greater in the lower body than in the upper body (Janssen, Heymsfield et al. 2000). Although multiple lifestyle behaviors and mechanisms contribute to the development of sarcopenia, the loss of lean body mass is the result of differences in rates of protein breakdown and protein synthesis.

### Section 3.1: Decreased Anabolic Signaling

The age related decrease in lean body mass may be a result of a combination of a decreased response to anabolic signaling and a reduction in anabolic stimuli. The anabolic response of skeletal muscle proteins to essential amino acids, which are mainly responsible for the postprandial increase in muscle protein synthesis (Volpi, Kobayashi et al. 2003), is reduced in the elderly (Visser, Deeg et al. 2003). Skeletal muscle of older individuals is also resistant to the anabolic action of insulin (Rasmussen, Fujita et al. 2006). Although the exact mechanism for the impaired response to both essential amino acids and insulin are not currently known, both appear to act through the mTOR pathway (Guillet, Prod'homme et al. 2004). The mTOR pathway will be discussed in more detail in the *nutrition, exercise and protein synthesis* section of this review. In the elderly, the blunted response to essential amino acids and insulin can result in reduced protein synthesis and therefore contribute to the reduction in lean body mass.

Aging is associated with several changes in hormonal levels, including a decrease in the concentrations of growth hormone (GH), insulin-like growth factor (IGF-1) and testosterone (Perrini, Laviola et al. 2010). Circulating GH levels decline after 30 years of age at a rate of approximately 1% per year, and as a result systemic IGF-1 concentrations also decline with advancing age (Hermann and Berger 2001). In skeletal muscle, GH promotes the fusion of myogenic precursor cells into existing myotubules, and this requires IGF-1 (Perrini, Laviola et al. 2010). IGF-1 concentrations are positively correlated with muscle protein synthesis rates, specifically myofibrillar protein and myosin heavy chain synthesis (Waters, Baumgartner et al. 2000). A sustained decrease in these hormones is associated with decreased muscle size and strength, diminished protein synthesis and increased apoptosis (Perrini, Laviola et al. 2010). Although GH and IGF-1 are involved in muscle protein metabolism and maintenance, there is conflicting evidence whether replacement is effective in maintaining or gaining muscle mass (Onder, Della Vedova et al. 2009). No effect on muscle strength, mass, or fiber size was observed following GH replacement (Lange, Andersen et al. 2002). Conversely, an increase of muscle mass and strength was shown in older individuals after three months of GH treatment (Welle, Thornton et al. 1996). The conflicting data involving the effectiveness of GH replacement may be the result of the duration of the treatment or dose, or it may be due to the extent of the initial GH deficiency.

Testosterone decreases gradually at a rate of 1% per year and bioavailable testosterone by 2% per year in males after 30 years of age (Morley, Kaiser et al. 1997). The overall reduction of testosterone is associated with loss of muscle strength and muscle mass (Mellstrom, Johnell et al. 2006). Replacement of testosterone increases muscle mass and increases hypertrophy of both types I and II fibers by down-regulating myostatin (Kovacheva, Hikim et al. 2010), which regulates myogenesis through inhibition of satellite cell activation, proliferation, and

differentiation. Additionally, testosterone decreases the age-related increase in oxidative stress and muscle cell apoptosis through suppression of c-jun NH2-terminal kinase and p21, respectively (Kovacheva, Hikim et al. 2010). However, there is conflicting research regarding testosterone's effect on muscle mass and strength gains following resistance exercise. Young men exposed to either basal testosterone concentrations or high testosterone concentrations experienced similar increases in muscle strength and muscle cross sectional area (West, Burd et al. 2010). Similarly, in post-menopausal women, there is conflicting evidence that hormone replacement therapy affects muscle mass and strength (Onder, Della Vedova et al. 2009). In older men and women, long term (1 year) treatment with either dehydroepiandrosterone (DHEA) or testosterone did not result in an increase in whole body or muscle protein synthesis (Henderson, Dhatariya et al. 2009).

Age related changes in neuromuscular signaling may also contribute to the loss of muscle mass. The number of  $\alpha$ -motor neurons declines with age (Brown 1972), resulting in denervation of the muscle fibers within the motor unit. This denervation causes the muscle fibers to atrophy, which leads to a decrease in muscle mass. However, the decline in  $\alpha$ -motor neurons is minor (only 10-15%), and therefore may not account for all deficits in neuromuscular signaling (Ulfhake, Bergman et al. 2000). Oxidative stress may also cause phenotypic changes in the motor neuron, which can interfere with neurotransmission and contribute to neurodegeneration (Ramirez-Leon, Kullberg et al. 1999). Overall, decreases in anabolic signaling and anabolic stimuli may result in decreased lean body mass in older individuals.

### Section 3.2: Oxidative Stress

Oxidative stress has been implicated as a central mechanism in the pathogenesis of sarcopenia (Semba, Lauretani et al. 2007). A significant age-dependent increase in oxidative damage to protein, lipids, and DNA occurs in skeletal muscle (Mecocci, Fano et al. 1999).

Oxidative stress can be the result of either a reduction in the antioxidant capacity or an increase in the production of reactive oxygen species (ROS), which is a byproduct of the electron transport chain within mitochondria (Fulle, Protasi et al. 2004). ROS activates nuclear factor-kappa B (NF- $\kappa$ B), which contributes to the loss of skeletal muscle mass (Li, Malhotra et al. 2008) through the following pathways: 1) NF- $\kappa$ B increases the expression of MuRF1 E3 ubiquitin ligase and other proteins in the ubiquitin proteasome, which leads to the degradation of skeletal muscle; 2) NF- $\kappa$ B induces muscle wasting by preventing myofilament protein synthesis and their organization into myofilament by down regulating MyoD; and 3) NF- $\kappa$ B also increases the expression of inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin 1 (IL1) and interleukin 6 (IL6) (Li, Malhotra et al. 2008). Proinflammatory cytokines (TNF- $\alpha$ , IL1 and IL6) promote muscle wasting directly by increasing myofibrillar protein degradation (Fong, Moldawer et al. 1989) and by decreasing protein synthesis (Lang, Frost et al. 2002). Furthermore, TNF- $\alpha$  induces expression of metalloproteinase-9 (MMP-9), a protease that degrades several components of extracellular matrix-cytoskeleton linkage in skeletal muscle (Li, Malhotra et al. 2008). Although oxidative stress contributes to protein breakdown, protein breakdown does not significantly increase with aging (Volpi, Sheffield-Moore et al. 2001); therefore, impaired protein synthesis would be expected to be responsible for the loss of muscle mass over time.

Within skeletal muscle, oxidative damage to mitochondrial DNA (mtDNA) contributes to sarcopenia. The mitochondria genome is especially susceptible to oxidative DNA damage due to multiple factors, including: 1) exposure of mtDNA to ROS as mtDNA are in close proximity to the electron transport chain (ETC), where complexes I and III are the predominant site for ROS production; 2) the lack of protective histones; 3) the lack of introns results in densely packed genetic information, so that damage will likely occur within a gene; and 4) a relative lack of DNA



repair enzymes (compared to nuclear DNA) (Hiona and Leeuwenburgh 2008). The ROS induced damage to the mtDNA gives rise to both mtDNA mutations and deletions that accumulate progressively during aging. The increased accumulation of mtDNA mutations is responsible for the observed decreases in the activities of complexes III and IV of the respiratory chain and the concomitant increase in complex II in older individuals (Chabi, Mousson de Camaret et al. 2005). The increase in complex II activity leads to a competition with complex I for the use of the quinone pool resulting in a reduced ability of complex I to oxidize NADH, which leads to mitochondria dysfunction, decreased energy production and muscle fiber atrophy (Cao, Wanagat et al. 2001). In the mitochondrial theory of aging, mitochondrial dysfunction leads to an even greater increase in ROS production, which results in further increases in oxidative stress and thus an increased rate of mtDNA damage and mutations (Hiona and Leeuwenburgh 2008). Mitochondrial dysfunction also impairs ATP production (Shigenaga, Hagen et al. 1994), which contributes to fatigue in older individuals. Furthermore, decreased efficiency of ATP production may result in a decrease in energetically costly processes such as protein synthesis (Dirks, Hofer et al. 2006). The overall effects of oxidative stress in skeletal muscle may lead to mitochondrial dysfunction, decreased protein synthesis and increased protein degradation, thus leading to reduced skeletal muscle mass and wasting.

### Section 3.3: Lifestyle Behaviors

Decreased physical activity may contribute to sarcopenia and wasting. Few older adults achieve the minimum recommended 30 or more minutes of moderate physical activity on five or more days per week. Data from the Centers for Disease Control and Prevention indicate that about 28-34% of adults aged 65 to 74 and 35-44% of adults ages 75 or older are inactive. Additionally, few older individuals engage in regular physical activity. Only 31% of individuals aged 65 to 74 reports participating in 20 minutes of moderate physical activity three or more

days per week, and only 16% report 30 minutes of moderate activity five or more days per week. For those aged 75 and older, levels of activity are even lower: 23% engage in moderate activity for 20 minutes three or more days per week and only 12% participate in such activity for 30 minutes five or more days per week. Exercise training could improve the ability of older individuals to remain functional and independent. Exercise can increase skeletal muscle size and strength (Charette, McEvoy et al. 1991), and can result in increases in whole body (Tarnopolsky, Atkinson et al. 1991) and muscle protein synthesis (Chesley, MacDougall et al. 1992). An 8-week training program (cycling exercise) in older persons can increase total energy expenditure by elevating resting metabolic rate, with up to a 12% increase in energy intake (Poehlman and Danforth 1991). A single bout of resistance exercise can increase ghrelin, an appetite stimulant, which can remain elevated 24 hours after exercise (Ghanbari-Niaki 2006). Therefore, exercise can increase both protein synthesis and food intake in older individuals.

The age associated physiologic reduction in food intake, which has been termed "the anorexia of aging", contributes to the low dietary energy intake that is common among healthy elderly adults (Fujita and Volpi 2004). Approximately 30% of older individuals consume the recommended dietary allowance (RDA) or less (McDowell, Briefel et al. 1994). Both physiological and nonphysiological causes result in this reduced consumption of food (and thus calories) with aging. The major physiological change that influences food intake is a loss of appetite. A number of factors are known to contribute to the decrease in appetite of older individuals including a decline in olfaction and gustaoception (Kaneda, Maeshima et al. 2000). These changes act together to decrease the perception of the hedonic qualities of food resulting in decreased enjoyment of food (Morley 2001) and a subsequent change in food preferences with an increased predilection for sweet, protein-poor foods (Morley 1997). Early satiation in older individuals also contributes to decreased food intake. When older individuals received the

same amount of food as younger persons they reported greater satiation (Clarkston, Pantano et al. 1997). Increased circulating cholecystokinin (CCK) and decreased rate of gastric emptying both contribute to this increased perceived satiation. Dementia, poor oral health, and swallowing disorders can also contribute to the decreased food intake (Morley 1997). Nonphysiological changes associated with aging also influence food intake. Many elderly experience social isolation caused either by living alone or due to the lack of social relationships and this social isolation may lead to reduced food consumption (McIntosh, Shiflett et al. 1989). Depression is more common in older individuals and approximately 90% of older individuals with depression lose weight compared to 60% in younger people with depression (Sheiham, Steele et al. 2001). The side effects of drugs are a major cause of weight loss in older individuals, as certain drugs are known to cause malabsorption and decrease appetite (Morley 1997). With the decrease in total food intake, older individuals are less likely to consume adequate vitamins and nutrients. In older individuals, vitamin D deficiency is associated with muscle weakness and sarcopenia (Visser, Deeg et al. 2003) and any vitamin deficiency may cause a reduction in defense mechanisms against free radicals, thus increasing oxidative stress (Weindruch 1995). In older individuals, various age related factors contribute to decreased food intake and negative energy balance.

#### **Section 4: Nitrogen Balance**

Nitrogen balance is commonly used to measure whole body protein balance. Protein is the major nitrogen-containing substance within the body, and a gain or loss of nitrogen can be considered as synonymous with a gain or loss of protein (Calloway and Spector 1954). When nitrogen intake exceeds the amount of nitrogen excreted, an individual is in positive nitrogen balance. Protein (nitrogen) intake that is less than the amount of nitrogen excreted results in a state of negative nitrogen balance and when protein intake is equal to the amount excreted, an

individual is in nitrogen balance. Nitrogen balance can be determined through the following equation:

$$\text{Nitrogen balance (g)} = \text{Nitrogen intake} - \text{nitrogen output}$$

Nitrogen intake is calculated from the amount of protein consumed, with 6.25 grams of protein equivalent to approximately one gram of nitrogen (Thomas, Bishop et al. 2007). Nitrogen output is defined as the nitrogen excreted in urine and feces plus miscellaneous nitrogen losses (Calloway, Odell et al. 1971). On average, 2 g/day of nitrogen is excreted in the feces and approximately 5 mg/kg body weight (bw)/day of nitrogen is lost through miscellaneous routes including sweat, hair, nails, and skin cells (Calloway, Odell et al. 1971).

The body disposes of nitrogen by converting ammonia to urea ( $(\text{NH}_2)_2\text{CO}$ ) through the urea cycle. Approximately 80% of excreted nitrogen is excreted as urea (Thomas, Bishop et al. 2007). In the liver, amino groups are transferred in transaminase reactions to glutamate. The glutamate can then be oxidized through the glutamate dehydrogenase reaction, forming  $\alpha$ -ketoglutarate and an ammonium ion ( $\text{NH}_4^+$ ). The  $\text{NH}_4^+$  is subsequently involved in the initial step of the urea cycle. The urea cycle begins with two mitochondrial reactions: 1) the condensation of  $\text{NH}_4^+$  and carbon dioxide ( $\text{CO}_2$ ) via carbamoyl phosphate synthetase to form carbamoyl phosphate and 2) condensation of carbamoyl phosphate with ornithine to form citrulline. Citrulline is then exported from the mitochondria and combines with aspartic acid to form arginosuccinate via the cytoplasmic arginosuccinate synthetase reaction. Arginosuccinate is then hydrolyzed to form fumarate and arginine. The cycle is completed when arginase cleaves arginine to form urea, which is then released into the blood and transported to the kidney for excretion (Storey 2004). The urea cycle is a vital process for eliminating nitrogen, as excess nitrogen in the body is highly toxic to the central nervous system (Roach and Benyon 2003).

Although nitrogen balance is commonly utilized to assess whole body protein turnover, there are limitations associated with the nitrogen balance technique. Nitrogen balance does not indicate whether a change in protein balance is a result of a change in protein synthesis or protein breakdown, or a combination of both. Additionally, changes in protein metabolism within individual tissues such as skeletal muscle can not be determined using the nitrogen balance technique. Inflated nitrogen retention is another limitation of the nitrogen balance technique (Kopple 1987). Nitrogen intake can be overestimated if any food is not completely consumed. Furthermore, nitrogen intake is estimated based on total protein intake, with 6.25 grams of protein containing 1 gram of nitrogen. However, 6.25 grams of protein do not always contain 1 gram of nitrogen, as 6.25 is an average based on protein quality. Inadequate collection of any excreted nitrogen can also contribute to the overestimated nitrogen retention. Collecting miscellaneous nitrogen losses can be quite challenging, and therefore relies on estimates. Thus, variability between individuals could contribute to the overestimated nitrogen balance. Finally, nitrogen balance requires a time period of approximately five to eight days for an individual to adjust to a change in total protein consumption (WHO 2007); therefore a short term controlled diet is required before nitrogen balance studies to allow an individual to adapt to a new protein intake (Rand, Young et al. 1976). However, recent research indicates that acute changes in protein intake ( $\pm 4\%$  of habitual protein intake) does not result in changes in whole body proteolysis or muscle protein synthesis (Yarasheski, Castaneda-Sceppa et al. 2011). Thus, a short term controlled diet may not be necessary for individuals to adapt to an acute change in protein intake.

Despite limitations, the nitrogen balance technique can be a useful technique for studying whole body protein turnover in aging populations. The contribution of muscle protein to whole body protein metabolism is significantly reduced in the elderly. Consequently, the

contribution of non skeletal muscle protein, especially visceral tissue whose rates of protein turnover are known to be more rapid, is proportionally greater with aging (Morais, Chevalier et al. 2006). Thus, the nitrogen balance technique with its noninvasive nature makes it a relatively easy and appropriate method to determine long term changes in whole body protein turnover in response to exercise and nutrition interventions in older individuals.

#### Section 4.1: Nutrition and Nitrogen Balance

Similar to protein synthesis, which was previously discussed, nitrogen balance is also affected by energy and protein intake. Nitrogen balance is better maintained when caloric intake is adequate, as a negative nitrogen balance results when energy intake is reduced by 15% (Todd, Butterfield et al. 1984). In addition, an individual in negative energy balance may decrease their physical activity to compensate for the inadequate caloric intake (Gorsky and Calloway 1983), which can further contribute to wasting. During caloric restriction, protein breakdown increases the availability of amino acids that are oxidized as energy and thus limits the availability of amino acids for protein synthesis. Increasing energy intake can result in a more positive nitrogen balance (Calloway and Spector 1954) as amino acids are utilized less for energy and are therefore available for protein synthesis. When energy intake is reduced, increased protein intake can enhance nitrogen retention (Calloway and Spector 1954). Older women in negative energy balance maintain more lean body mass when protein intake is increased from 0.8 g/kg bw to 1.6 g/kg bw per day (Layman, Boileau et al. 2003). Similarly, elderly women in energy balance maintain a significantly greater nitrogen balance when a high protein diet (0.92 g/kg bw per day) is consumed compared to a low protein diet (0.45 g/kg bw per day). Furthermore, the low protein diet also resulted in a significant loss of lean body mass, muscle function and immune function (Castaneda, Charnley et al. 1995). Inadequate protein intake limits the availability of amino acids for protein synthesis and thus a negative nitrogen

balance results. When caloric intake is adequate, protein intake significantly above the RDA does not result in significant increases in nitrogen balance (Calloway and Spector 1954) as excess nitrogen is excreted via the urea cycle. In general, total energy intake required to maintain nitrogen balance decreases as protein consumption increases.

#### Section 4.2: Exercise and Nitrogen Balance

Both aerobic and resistance exercise can result in a more positive nitrogen balance by increasing nitrogen retention. If energy balance is maintained, one hour of aerobic exercise can significantly increase nitrogen balance and spare approximately 2.5 mg/kg bw of nitrogen (Todd, Butterfield et al. 1984). Aerobic exercise can also increase nitrogen balance when caloric intake is inadequate, but the increase is less pronounced compared to when energy balance is maintained (Todd, Butterfield et al. 1984). However, nitrogen balance is better maintained during a 15% negative energy balance when the energy deficit is a result of exercise, rather than a reduction in caloric intake (Todd, Butterfield et al. 1984). Similar to aerobic exercise, resistance exercise can also result in a more positive nitrogen balance. When older adults in a 12 week resistance exercise program consumed a negative energy balance diet that provided either 0.8 g/kg bw (low protein) or 1.6 g/kg bw (high protein) of protein, individuals on the high protein diet retained more nitrogen than the low protein diet (Campbell, Crim et al. 1995). Although exercise can compensate (to a point) for inadequate energy and protein intake, nitrogen retention is better maintained when both energy and protein intake are adequate.

### **Section 5: Nutrition, Exercise, and Protein Synthesis**

#### Section 5.1: mTOR Regulates Protein Synthesis

mTOR is a nutrient and energy-sensing protein that regulates protein synthesis. mTOR stimulates protein synthesis mainly through three downstream effectors that are involved in protein translation, including: eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), p70

ribosomal S6 kinase ( $p70^{s6k}$ ), and eukaryotic initiation factor 4G (eIF4G) (Raught, Gingras et al. 2001). mTOR controls various components involved in the initiation and elongation stages of translation, both of which are implicated as the rate limiting step in protein synthesis under conditions of fasting (Rannels, Pegg et al. 1978) and feeding (Morgan, Jefferson et al. 1971), respectively. When 4E-BP1 is phosphorylated, eIF4E is released and can then form the eIF4F complex together with eIF4G. The assembly of this complex is necessary for translation initiation (Raught, Gingras et al. 2001).  $p70^{s6k}$  increases the activity of eEF2, which mediates the translocation during the elongation phase of translation (Browne, Finn et al. 2004). Although the mechanism by which mTOR activates its effectors is not fully understood, mTOR could stimulate 4E-BP1, eIF4G, and  $p70^{s6k}$  either by direct phosphorylation, indirectly by activating another protein kinase (Raught, Gingras et al. 2001), or by inhibiting a phosphatase (Li, Corradetti et al. 2004).

Energy status can regulate the rate of protein synthesis through mTOR signaling. AMPK functions as a sensor of energy status, which can then mediate its effects through mTOR. (Miyazaki and Esser 2009). AMPK is regulated by changes in the AMP to ATP ratio, such that an increase in the AMP to ATP ratio stimulates AMPK. Activated AMPK phosphorylates tuberous sclerosis complex 2 (TSC2) and thus enhances its inhibitory function leading to decreased mTOR activity (Inoki, Zhu et al. 2003). In energy deprived conditions, further phosphorylation by glycogen synthase kinase 3 (GSK-3) on multiple residues of TSC2 leads to further inhibition of mTOR activity (Inoki, Ouyang et al. 2006). During times of energy surplus, such as feeding, mTOR can stimulate protein synthesis in response to insulin. Insulin stimulates protein synthesis by activating the insulin signaling pathway leading to an increase in phosphoinositide 3-kinase (PI3K) and Akt/PKB (protein kinase B) activity. Akt/PKB phosphorylates and inhibits the TSC2, thus allowing ras homolog enriched in brain (Rheb) to accumulate in its active GTP-bound form,



which acts to stimulate mTOR and increases protein synthesis (Avruch, Hara et al. 2006). Meanwhile, during an energy surplus, the ratio of AMP to ATP decreases and the AMPK inhibition of mTOR decreases, allowing for further stimulation of mTOR and protein synthesis.

mTOR is regulated by the availability of amino acids. Amino acid deprivation leads to decreased mTOR signaling and decreased rates of protein synthesis. The decreased mTOR signaling is reversed within minutes by the re-addition of amino acids (Hara, Yonezawa et al. 1998). Essential amino acids alone can stimulate protein synthesis, with leucine having the most potent effect (Anthony, Yoshizawa et al. 2000). The mechanism by which amino acids regulate mTOR signaling is not currently known, although it is believed that the amino acids can inhibit an upstream phosphatase, stimulate a kinase, interact with various upstream proteins that act on mTOR or amino acids can directly activate mTOR (Deldicque, Theisen et al. 2005). Amino acids are required for translation, and therefore when amino acid availability is low, mTOR signaling decreases and consequently protein synthesis is also decreased.

Exercise can also enhance protein synthesis through the mTOR pathway. The mechanism of mTOR activation is unclear, but it is believed to occur through activation of PKB or through direct phosphorylation of mTOR (Deldicque, Theisen et al. 2005). Exercise induced p70<sup>S6k</sup> activity is strongly correlated with increased skeletal muscle mass after six weeks of resistance training (Baar and Esser 1999). Additionally, nutrition can take advantage of the anabolic period triggered by exercise. Consumption of a branched chain amino acid beverage during exercise significantly increases phosphorylation (and thus activation) of mTOR (Liu, Jahn et al. 2001; Karlsson, Nilsson et al. 2004). mTOR regulates protein synthesis in response to exercise, nutrition and the energy state of an individual.

### Section 5.2: Timing of Intake

The timing of nutrient intake relative to exercise is important to enhance protein synthesis. A mixed nutrient supplement taken immediately after one hour of moderate-intensity aerobic exercise results in a greater increase in whole body protein synthesis compared to ingestion three hours after exercise (Levenhagen, Gresham et al. 2001). Similarly, older individuals in a 12-week resistance training program had greater increases in skeletal muscle cross sectional area and strength when a mixed nutrient supplement was consumed immediately after resistance exercise compared to two hours after exercise (Esmarck, Andersen et al. 2001). In another study, young female athletes in exercise-induced negative energy balance consumed either a mixed-meal beverage or a non-caloric placebo beverage after exercise. Diet was replicated between the two trials, so that the timing of intake was the only difference between the trials. Although nitrogen balance was not significantly greater when the mixed-meal beverage was consumed post-exercise, there was a strong trend ( $p=0.06$ ) for an increase in nitrogen balanced when the mixed-meal beverage was consumed post-exercise (Roy, Luttmer et al. 2002). Moreover, when older individuals in energy balance consumed a chocolate milk beverage immediately after one hour of aerobic exercise rather than earlier in the day, a more positive nitrogen balance was maintained (Jordan, Melanson et al. 2010). However, there is conflicting data about the effect of timing of feeding. An amino acid-carbohydrate drink consumed one or three hours after resistance exercise had similar increases in muscle protein synthesis (Rasmussen, Tipton et al. 2000). Furthermore, the response of net muscle protein synthesis to consumption of an amino acid-carbohydrate solution immediately prior to resistance exercise is greater compared to after exercise (Tipton, Rasmussen et al. 2001). Yet, in another study, an amino acid-carbohydrate supplement ingested before resistance exercise did not enhance post-exercise muscle protein synthesis compared to exercise without added

nutrients (Fujita, Dreyer et al. 2009). Since nitrogen balance is closely related to energy balance (Todd, Butterfield et al. 1984), the conflicting data regarding timing of nutrient intake may be due to differences in energy balance. Despite conflicting research, consuming protein immediately after exercise, while in energy balance, appears to be most effective for enhancing protein synthesis.

### Section 5.3: Protein Quality

The quality of proteins consumed after exercise can affect protein synthesis. Protein quality depends on both the amino acid composition and the digestibility of the proteins. A high quality protein, also known as a complete protein, contains adequate amounts of all essential amino acids, whereas an incomplete protein, or low quality protein is lacking in one or more essential amino acids. Sources of complete proteins are mostly foods derived from animal origin such as milk and meat, while incomplete proteins are usually derived from plant foods including vegetables, legumes and grains (Gropper, Smith et al. 2009). In elderly women, consuming a high-protein meal from an animal source, compared to a high-protein meal from non-animal sources, resulted in a significant increase in net protein synthesis, which over time could result in maintenance of lean body mass (Pannemans, Wagenmakers et al. 1998). Similarly, consumption of milk-based proteins resulted in a positive net protein balance and an increased rate of muscle protein synthesis after resistance exercise compared to soy-based proteins (Wilkinson, Tarnopolsky et al. 2007). The difference in the metabolism of milk and soy proteins is due to the rate of digestion (Bos, Metges et al. 2003). The rate of digestion and absorption of dietary amino acids varies according to the type of ingested dietary protein. For example, milk consists of both whey and casein proteins (Jenness 1979), whereas soy contains a single protein fraction that is digested similar to whey protein (Bos, Metges et al. 2003). Whey protein, which is considered a “fast” protein, is rapidly digested, and the amino acids are quickly

absorbed resulting in rapid appearance of amino acids in the plasma. The rapid increase in plasma amino acids results in an acute increase in protein synthesis (Boirie, Dangin et al. 1997). Conversely, casein protein, which is considered a “slow” protein, is digested more slowly, and the plasma appearance of amino acids is slower and more prolonged. Ingestion of casein proteins results in a minor increase in protein synthesis and a significant decrease in protein breakdown (Boirie, Dangin et al. 1997). In young men at rest, muscle protein synthesis was 93% greater after consumption of whey compared to casein and 18% greater than soy. Furthermore, following resistance exercise muscle protein synthesis was 122% greater after consumption of whey compared to casein and 31% greater than soy (Tang, Moore et al. 2009). Consumption of milk, which consists of both whey and casein proteins, could maximize net protein accretion by stimulating protein synthesis and reducing protein breakdown.

In general, compared to lower quality proteins, high quality proteins such as milk have increased essential amino acid content and in particular, increased leucine content. Leucine is a potent stimulator of protein synthesis and can stimulate protein synthesis in muscle independent of other amino acids (Buse and Reid 1975) or insulin (Anthony, Reiter et al. 2002). Leucine stimulates protein synthesis by enhancing translation initiation through the mTOR pathway by down-regulating the translational repressor 4E-BP1 (Anthony, Anthony et al. 2000) and up-regulating p70<sup>s6k</sup> (Burnett, Barrow et al. 1998). Consumption of a leucine-rich supplement after exercise resulted in a significant increase in muscle protein synthesis compared to both a carbohydrate only supplement and a carbohydrate with added protein supplement (Koopman, Wagenmakers et al. 2005). Thus, consuming high quality proteins with adequate leucine after exercise can increase lean body mass.

#### Section 5.4: Management of Wasting with Exercise and Nutrition

Exercise and nutrition represent two strategies to maintain lean body mass and prevent wasting. Older men in a 12-week resistance training program increased muscle cross sectional area more than 11% and improved leg extensor and flexor strength by 107% and 226%, respectively (Frontera, Meredith et al. 1988). Similarly, older women in a 12-week aerobic training program increased whole muscle volume by 12% and increased leg extensor strength by 55% (Harber, Konopka et al. 2009). Therefore, aerobic and resistance exercise can increase both strength and lean body mass in older individuals. Timing nutritional intake after exercise can further enhance the beneficial effects of exercise. When older individuals consumed a chocolate milk beverage immediately after one hour of aerobic exercise rather than earlier in the day, a more positive nitrogen balance was maintained (Jordan, Melanson et al. 2010). In addition, when nutrition is combined with exercise, muscle protein synthesis can remain elevated for up to 72 hours after exercise (Miller, Olesen et al. 2005). Thus, exercise followed by a high quality protein source could further enhance protein synthesis and lean body mass more than exercise or nutrition alone.

In addition to increased muscle size and strength, exercise can also increase appetite and enhance total caloric intake (Ghanbari-Niaki 2006). By increasing appetite and energy intake, older individuals are more likely to consume adequate vitamins and nutrients, and remain in energy balance. Energy balance is important for maintaining lean body mass as negative energy balance results in a more negative nitrogen balance, and therefore a reduction in whole body protein synthesis (Hoffer, Bistran et al. 1984; Todd, Butterfield et al. 1984). Adequate intake of vitamins is essential for preventing sarcopenia, as any vitamin deficiency may cause a reduction in defense mechanisms against free radicals, which increases oxidative

stress (Weindruch 1995). As discussed previously, oxidative stress is a central mechanism in the pathogenesis of sarcopenia.

Protein intake above the RDA (0.8 g/kg bw) may be another approach to prevent sarcopenia and wasting. In elderly women, protein intake below the RDA is associated with significant losses of muscle mass and muscle strength (Castaneda, Charnley et al. 1995), however almost 40% of individuals over the age of 70 do not consume the RDA for protein (Houston, Nicklas et al. 2008). A study by Campbell et al. demonstrated that even when older individuals in energy balance consume the RDA for protein, negative nitrogen balance and loss of mid-thigh muscle area results (Campbell, Trappe et al. 2001), which indicates that protein intake above the RDA may be necessary to maintain nitrogen balance. However, recent research from their lab indicates that the current RDA for protein intake is adequate for older individuals to maintain nitrogen balance (Campbell, Johnson et al. 2008). Despite early research indicating that the RDA for protein intake may be inadequate, recent research indicates that the current RDA for protein is adequate for older individuals to maintain nitrogen balance.

The age related decrease in muscle mass and function is common as individuals' age. Sarcopenia is a multifactorial process that is associated with a retraction of anabolic signaling and an increase in catabolic stimuli. Although multiple lifestyle behaviors and mechanisms contribute to the development of sarcopenia, the loss of lean body mass is the result of differences in rates of protein breakdown and protein synthesis. Over time, if protein breakdown exceeds protein synthesis, loss of lean body mass will occur. Sarcopenia is a gradual process, so any intervention that slightly increases nitrogen balance and thus protein synthesis may be adequate to prevent or reduce the loss of lean body mass. Overall, few strategies exist to prevent or treat sarcopenia, but nutrition and exercise interventions appear to be ideal for managing sarcopenia. Our lab previously showed that timing protein intake after aerobic

exercise versus earlier in the day, can increase nitrogen balance in older individuals in energy balance. Since caloric deficiencies are common in older individuals, this study aims to investigate whether protein consumption after exercise, rather than earlier in the day, can increase nitrogen balance in older individuals in negative energy balance.

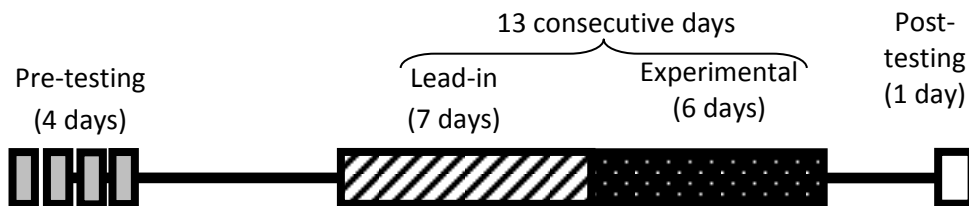
## CHAPTER 3

### METHODS AND PROCEDURES

#### Study Overview

The current study investigated whether consumption of protein immediately after one hour of moderate aerobic exercise, compared to earlier in the day, can improve nitrogen balance in older individuals in negative energy balance. The study included four separate phases, which included: pre-experimental testing, a seven-day lead in diet, a six-day experimental phase, and a one-day post-testing period (Figure 3.1).

Figure 3.1 Study timeline



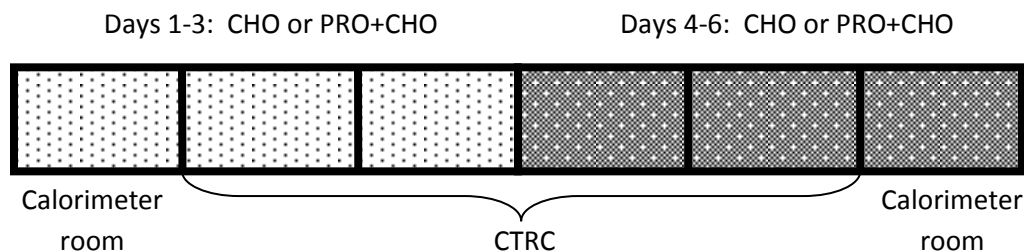
Pre-testing		Lead-in (outpatient)	Experimental (inpatient)		Post-testing
Day 1: GXT, diet log collection Day 2: RMR, DEXA Day 3: $VO_{2max}$ Day 4: steady-state $VO_2$	2-3 week break to allow for diet planning and in-patient scheduling	Controlled diet	Controlled diet, 1-hr daily exercise followed by test beverage, 24-hr urine collection, daily weight measurement, activity monitor, 2 days in Cal room	~1 wk	DEXA, weight measurement



During the pre-testing phase of the study, data was collected that was used for the planning of the diets (lead-in and inpatient diets) and to determine the relative intensity of the daily aerobic exercise during the inpatient phase. The seven-day lead-in diet allowed subjects to adapt to the level of protein that subjects were exposed to during the inpatient phase of the study.

The inpatient phase of the study consisted of a 6-day inpatient stay at the University of Colorado Denver Clinical and Translational Research Center (CTRC). Subjects completed two, three-day trials in a randomized cross-over design (CHO and PRO+CHO). Subjects stayed one day during each three-day trial (inpatient Day 1 and Day 6) in a whole room calorimeter to confirm that subjects were in 15% negative energy balance (see Figure 3.2).

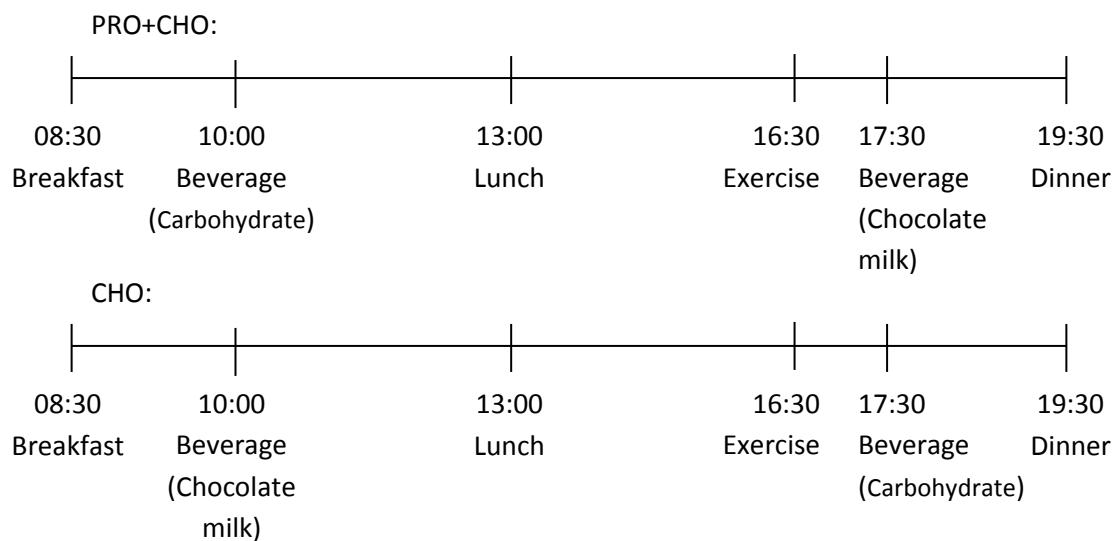
Figure 3.2 Inpatient phase



Subjects stayed in a hospital room in the CTRC during days two through five, where they were permitted to leave twice a day for 30 minutes. For the duration of the stay, breakfast, lunch, and dinner were delivered at the same specific time each day. One hour of cycling exercise was performed each day from 16:30 until 17:30. Immediately following the exercise, subjects consumed their post-exercise beverage. During the PRO+CHO condition, subjects consumed a chocolate milk beverage immediately following the exercise and during the CHO condition, a carbohydrate beverage was consumed immediately post-exercise. The diets for

each three-day condition were isocaloric and isonitrogenous. In addition, the foods consumed during each condition were identical and consumed at similar times for each condition. Only the timing of the protein-containing beverage differed between the two conditions. The study protocol was approved by the Colorado State University Institutional Review Board and the Colorado Multiple Institutional Review Board for human participants' research.

Figure 3.3 Timing of meals and beverages during inpatient phase



### Participants

Ten male (n=2) and female (n=8) subjects between 55 and 75 years old were recruited for the study from the Fort Collins and Loveland Colorado area. For inclusion, individuals were required to be non-smoking, inactive, lactose tolerant, and not taking any medications. Other exclusion criteria included: obesity (BMI > 30), any orthopedic injury that would impede their ability to exercise, any condition that affected food digestion or digestion, a thyroid condition (TSH <0.05uU/mL or TSH>5.0uU/mL), a bleeding disorder, or any current illness or infection. See Table 3.1 for participant characteristics.

Table 3.1 Participant characteristics

	Male (n=2)	Female (n=8)	All subjects (n=10)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
Age	67.0 $\pm$ 1.0	63 $\pm$ 1.8	63.8 $\pm$ 1.7
Height (cm)	183.4 $\pm$ 1.0	165.3 $\pm$ 2.4	168.9 $\pm$ 3.2
Weight (kg)	92.2 $\pm$ 1.0	60.9 $\pm$ 1.7	67.2 $\pm$ 4.4
BMI (kg/m <sup>2</sup> )	27.4 $\pm$ 0.3	22.3 $\pm$ 0.6	23.6 $\pm$ 0.8
Body fat (%)	27.9 $\pm$ 1.0	34.5 $\pm$ 1.7	33.2 $\pm$ 1.7
VO <sub>2max</sub> (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	32.2 $\pm$ 0.5	28.5 $\pm$ 1.7	29.2 $\pm$ 1.4

### Screening

The initial screening visit was conducted at the Human Performance Clinical/Research Laboratory (HPCRL) at Colorado State University. During the screening visit, subjects completed an informed consent, a HIPAA-B Approval form, and a medical and exercise history questionnaire (Appendices I, II, and III). In addition, an online food preference and food allergy questionnaire from the CTRC was completed and three-day diet logs were given to the subjects. Subjects were instructed to return their completed three-day diet logs at their first pre-testing appointment. All foods from three-day diet records were entered into Nutrition Pro software (Axxya Systems, Stafford, TX) and analyzed to determine habitual (free-living) energy and macronutrient intake. In addition, subjects also had a blood draw at Poudre Valley Hospital prior to their initial pre-testing visit to ensure that thyroid stimulating hormone concentrations were within 0.05uU/mL to 5.0uU/mL.

### Pre-Testing

Over four separate days, subjects completed a series of pre-testing at the HPCRL. During the initial visit, subjects completed a graded exercise test (GXT) on a treadmill. The test began with subjects walking at 3.3 miles per hour with a 0.0% grade. The treadmill speed remained at 3.3 miles per hour for the duration of the test, but the grade increased by 2% after

the first minute and by 1% every minute after the first minute. During the test, heart rate, rating of perceived effort, blood pressure, and an electrocardiograph (EKG) was recorded every three minutes. The test was ended when subjects reached 85-100% of their predicted maximum heart rate ( $220 - \text{age}$ ) or when the subjects were too fatigued to continue. The GXT was supervised by a cardiologist and subjects were excluded if the tests indicated an ischemic or hypertensive response to the exercise.

On a different day subjects returned to the HPCRL after an overnight fast. Resting metabolic rate (RMR) (Parvomedics TrueOne 2400, Sandy, UT) was measured to determine 24-hour resting caloric expenditure. During the test, subjects rested in the supine position with the lights dimmed and all expired gases were collected. Subjects were instructed to remain still and to refrain from sleeping. The flow rate was adjusted to maintain  $\text{FECO}_2$  levels between 0.9-1.0%. The first 15 minutes of the test was used to achieve the appropriate flow rate and to allow the subjects to become familiarized with the experimental conditions. The data from the final 30 minutes of the test was used to predict RMR. The values for daily energy expenditure were averaged and any measurement outside of  $\pm 2$  standard deviations was omitted. After the RMR test was completed subjects underwent a dual-energy X-ray absorptiometry (DEXA) scan (QDR 4500W, Hologic, Inc., Bedford MA) to determine body composition.

On the third day of testing subjects completed an incremental exercise test on a cycle ergometer (Monark Excalibur, Groningen, The Netherlands) with indirect calorimetry (Parvomedics TrueOne 2400, Sandy, UT) to determine  $\text{VO}_{2\text{max}}$ . Subjects pedaled at 50 Watts for the first minute, and Watts increased by 20 for females and 30 for males every two minutes thereafter. Throughout the test, subjects maintained a pedal rate between 70-90 rpm's. The test was stopped when subjects reached volitional exhaustion. Subjects were considered to have reached volitional exhaustion when  $\text{VO}_2$  reached a plateau (less than 2mL per kg per min

increase) or when heart rate was within 10 beats of predicted maximum heart rate and respiratory exchange ratio (RER) was greater than 1.1. After  $\text{VO}_{2\text{max}}$  was determined, 55% of each subject's  $\text{VO}_{2\text{max}}$  was calculated. Equation 3.1 was used to estimate the cycle ergometer rate that would correspond to a steady-state exercise intensity of 55% of  $\text{VO}_{2\text{max}}$ .

Equation 3.1 ACSM leg cycle ergometry equation

$$\text{VO}_2 (\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}) = 1.8 (\text{work rate}) / (\text{BM})^* + \text{resting VO}_2 (3.5 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}) + \text{unloaded cycling } (3.5 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})$$

$$*\text{work rate} = \text{kg} \cdot \text{m} \cdot \text{min}^{-1} \text{ and } \text{BM} = \text{body mass (kg)}$$

On another day subjects returned to the HPCRL for a submaximal steady-state cycling test. Subjects began cycling at their estimated workload (from equation 3.1) and once  $\text{VO}_2$  stabilized they continued cycling for an additional 30 minutes. Through indirect calorimetry, energy expenditure was determined by averaging the energy expenditure from the final 30 minutes of cycling. The calculated energy expenditure from the 30 minutes of cycling was used to estimate the subject's energy expenditure during the one hour of cycling during the inpatient period.

Lead-in Diet

Following completion of the pretesting, subjects completed a controlled 7-day lead-in diet. The lead-in diet allowed subjects to adapt to a new level of protein intake, so that nitrogen balance measurements made during the inpatient stay would not reflect an acute adaption to a new level of protein intake. During the lead-in diet period, subjects arrived every morning at the Colorado State University Nutrition Center for breakfast. Participants ate breakfast at the nutrition center under the supervision of study staff. Food for the remainder of the day was prepared and given to the subjects in a cooler. Subjects were instructed to eat only the food given to them by the study staff and to eat all of the food given to them. If the subjects were

unable to eat any of the food provided to them, they brought the food back with them the following day so that it could be weighed. Subjects were instructed to continue their typical daily activities throughout the duration of the 7-day lead-in period.

The lead-in and inpatient diets followed United States Department of Agriculture (USDA) nutritional guidelines (UnitedStates.Dept.Agriculture 2005) and were constructed using Pronutra software (Viocare, Inc., Princeton, NJ). The lead-in diets (and all inpatient diets) macronutrient breakdown was: 15% protein, 30% fat, and 55% carbohydrate expressed as a percentage of total calories. Subjects remained in energy balance for the duration of the lead-in diet. Total energy intake was calculated using each subject's RMR, multiplied by an activity factor of 1.55, which approximates the activity levels of free living sedentary elderly individuals (Pannemans and Westerterp 1995).

#### Inpatient period

The inpatient period involved a 6-day stay at the University of Colorado-Denver CTRC. Days 1 and 6 of the inpatient stay were spent in a 12' x 12' whole room calorimeter, while days 2-5 were spent in a regular inpatient room at the CTRC. During days 2-5, subjects were permitted to leave their room twice a day for 30 minutes. During the inpatient stay, subjects were not permitted to perform any exercise other than the one hour of prescribed cycling. Subjects were only allowed to eat the food that was provided to them by study staff. Subjects were permitted to consume water ad libitum, and were allowed to request additional non-caloric, non-caffeinated beverages. Blood samples were obtained in the fasted state on Days 1, 3, and 6. Subjects were weighed in their hospital gowns on the same scale each morning.

Each subject completed two, 3-day trials in a randomized crossover design. Every day at 16:30, subjects completed one hour of cycling exercise at 55% of their  $VO_{2max}$ . The exercise was performed on a Lode Corival bicycle ergometer (Lode, Groningen, The Netherlands). Subjects

recorded their heart rate every 15 minutes during exercise using a heart rate monitor (Polar FS1, Lake Success, NY). The exercise was intended to simulate a brisk walk, which was well tolerated by all subjects.

Immediately following the daily exercise bout, a post-exercise beverage was consumed. During the PRO+CHO phase, a 248-kcal chocolate milk drink which contained 15.3 g protein, 43.6 g carbohydrate, and 1.3 g fat (330g skim milk, 4.0g whey protein, and 42g chocolate syrup) was consumed immediately post-exercise. During the CHO phase a 247-kcal carbohydrate beverage, which contained 0.0g protein, 63.51g carbohydrate, 0.06g fat was consumed immediately post-exercise. During the PRO+CHO trial, the CHO beverage was consumed as a snack at 10:00 and during the CHO trial, the PRO+CHO beverage was consumed as a snack at 10:00. The order in which subjects completed each trial was randomized.

The diets for each 3-day trial were reproduced and identical in caloric intake, macronutrients, and foods consumed. The diet plans for Day 1, 2, and 3 were repeated on Day 6, 4, and 5, respectively (Table 3.2 provides a sample inpatient diet plan). The only difference between trials was the timing of intake of the protein beverage. Similar to the lead-in diet, the inpatient diet's percentage of total kilocalories for each macronutrient was: 55% carbohydrate, 30% fat, and 15% protein. All study diets corresponded with the USDA's acceptable macronutrient distribution ranges (10-35% protein, 20-35% fat, and 45-65% carbohydrate) (UnitedStates.Dept.Agriculture 2005).

Table 3.2 Sample inpatient diet plan

Day	Energy intake (kcal)	Diet: Breakfast (B), Snacks (S), Lunch (L), Dinner (D)
1 (CHO) Cal room	1896	B-oatmeal S- chocolate milk L-salad S- CHO beverage D-spaghetti
2 (CHO)	1988	B-toast S- chocolate milk L-soup S- CHO beverage D-stir-fry
3 (CHO)	1972	B-eggs S-chocolate milk L-sandwich S-CHO beverage D-steak/potato
4(PRO+CHO)	1988	B-toast S- CHO beverage L-soup S- chocolate milk D-stir fry
5(PRO+CHO)	1972	B-eggs S-CHO beverage L-sandwich S-chocolate milk D-steak/potato
6(PRO+CHO) Cal room	1896	B-oatmeal S- CHO beverage L-salad S- chocolate milk D-spaghetti

In order to plan the diets for the inpatient period, total daily energy expenditure was estimated. An activity factor of 1.30-1.35 was multiplied by the subjects RMR for the room calorimeter days, and an activity factor of 1.40-1.45 was multiplied by the subjects RMR for the non-calorimeter days (Days 2-5) (Jordan, Melanson et al. 2010). A lower activity factor was used for calorimeter days because activity levels are reduced when confined to the calorimeter room. Exercise energy expenditure (EE) was estimated from the steady-state  $VO_2$  data (measured during pre-testing) and an additional 20% of exercise calories were added in order to account for excessive post-exercise oxygen consumption (EPOC) (Melanson, Gozansky et al. 2009). Equation 3.2 was used to predict total daily energy expenditure (TDEE) for calorimeter room and non-calorimeter days.



### Equation 3.2 Estimation of TDEE

$$EE = (\text{Activity factor} \times \text{RMR}) + (\text{exercise EE} + (0.2 \times \text{exercise EE}))$$
 (Gersovitz, Motil et al. 1982).

The calorimeter room was 12' x 12' and contained a bed, sink, toilet, bicycle ergometer, computer, and television. Subjects entered the calorimeter room at 07:45 on Days 1 and 6, and exited at 07:15 on following morning. To prevent air from escaping, all meals were passed through an air lock that could not be simultaneously opened from the inside and outside. Daily energy expenditure and substrate oxidation were calculated by the difference in gas content that was entering and exiting the room. Since air was exiting the room for analysis, known concentrations of O<sub>2</sub> and CO<sub>2</sub> were continuously pumped into the calorimeter room. The difference in gas concentrations in the incurrent and excurrent airstreams was measured using a differential paramagnetic oxygen analyzer (Siemens Oxymat 6E Oxygen Gas Analyzer; Siemens, Houston, TX) and a differential infrared carbon dioxide analyzer (ABB Advance Optima Uras 14 NDIR CO<sub>2</sub> Analyzer; ABB, Zurich, Switzerland). All analyzed gas values were corrected for temperature, barometric pressure, and relative humidity (Melanson, Ingebrigtsen et al. 2010). Total energy expenditure and substrate oxidation were calculated using oxygen consumption and respiratory quotient (Jequier, Acheson et al. 1987). All measurements were one-minute averages.

During the inpatient stay, subjects wore an accelerometer (Actigraph GT1M, Pensacola, FL), which was removed during exercise, sleep, and showering. Using equations 3.2 and 3.3, energy expenditure from activity was estimated from the accelerometer. The Freedson equation (Equation 3.3) was used to estimate energy expenditure for activity counts >1,952 per minute. For activity counts ≤ 1,952 per minute, energy expenditure was estimated using the work energy theorem (Equation 3.4)

Equation 3.3 Freedson equation (Freedson, Melanson et al. 1998)

$$\text{Kcal/min} = 0.00094 * \text{counts/minute} + 0.1346 * \text{body mass (kg)} - 7.37418$$

Equation 3.4 Work-energy theorem

$$\text{Kcal/min} = 0.0000191 * \text{counts/minute} * \text{body mass (kg)}$$

TDEE on calorimeter days was directly measured and non-calorimeter days were estimated using equation 3.5.

Equation 3.5 TDEE on non calorimeter days

$$\text{TDEE} = \text{RMR} + \text{non-exercise EE (from accelerometer)} + \text{exercise EE (measured as average of 2 days in calorimeter room)} + \text{dietary induced thermogenesis (measured as average of two days in calorimeter room)}$$

Energy balance (EB) for the inpatient period was determined using Equation 3.6, with energy intake calculated from the dietician.

Equation 3.6 Daily energy balance

$$\text{EB} = \text{caloric intake} - \text{TDEE}$$

Daily physical activity level (PAL) was calculated using Equation 3.7, while non-exercise PAL was calculated using equation 3.8. The value for exercise energy expenditure was multiplied by 1.2 to account for the additional energy expenditure resulting from EPOC (Melanson, Gozansky et al. 2009).

Equation 3.7 Physical activity level (PAL)

$$\text{PAL} = \text{TDEE} / \text{RMR}$$

Equation 3.8 Non-exercise PAL.

$$\text{Non-exercise PAL} = (\text{TDEE} - \text{Exercise EE} * 1.2) / \text{RMR}$$

### Nitrogen Balance

Twenty-four hour urine samples were collected to determine urinary nitrogen. Urine was collected in acid and total volume was measured. Two 10-ml aliquots per 24-hour collection were frozen and stored for later analysis. Nitrogen was analyzed using an Antek 7000 Elemental Nitrogen Analyzer (PAC, Houston, TX). Nitrogen balance was calculated using equation 3.9. Nitrogen intake was calculated as (protein intake (g)/6.25) since 6.25 grams of protein contains on average 1 gram of nitrogen. Nitrogen output is defined as the nitrogen excreted in urine and feces plus miscellaneous nitrogen losses (Calloway, Odell et al. 1971). Daily miscellaneous nitrogen losses were estimated at 5 mg/kg bw, and fecal nitrogen losses were estimated at 2 grams (Calloway, Odell et al. 1971).

### Equation 3.9 Nitrogen balance

$$\text{Nitrogen balance (g)} = \text{Nitrogen intake} - \text{nitrogen output}$$

### Post-testing

Approximately one week after the inpatient period, subjects returned to the HPCRL for a final DEXA scan and weight measurement.

### Statistical Analysis

All statistical analysis were done using GraphPad Prism (version 4.00 for Macintosh, Graphpad Software, San Diego, California). Nitrogen balance data, energy balance data, pre and post body weight, pre and post fat free mass, and pre and post body fat were analyzed using student's paired *t*-tests. Pearson's correlation coefficients (*r*) were used to determine any correlations between nitrogen balance and energy balance, or nitrogen balance and relative protein intake. One-way repeated measures ANOVA analyzed diet-related variables within free-living, lead-in and inpatient diets. Any differences within the diet-related variables were determined using the Student Newman-Keuls post-hoc test. All variables that were tested have

a level of significance of  $p < 0.05$ . All data is presented as the mean  $\pm$  the standard error of the mean.

## CHAPTER 4

### RESULTS

#### Energy and macronutrient intake

All study diets followed USDA Nutritional guidelines and were designed based on each subjects' personal preferences. Total caloric intake during free-living (as reported from 3-day diet records), lead-in, and inpatient periods are depicted in Figure 4.1. Total caloric intake was not significantly different between free-living, lead-in, and inpatient diets. The decrease in total caloric intake during the inpatient period reflects the designed 15% negative energy balance for each subject. Figure 4.2 depicts the average macronutrient intakes for subjects under the free-living, lead-in, and inpatient periods of the study. Macronutrient intake was not significantly different between free-living, lead-in, and inpatient diets. Similarly, the decrease in energy intake during the inpatient period of the diet reflects the 15% negative energy balance of each subject.

Figure 4.1 Energy intake for free-living, lead-in, and inpatient diets

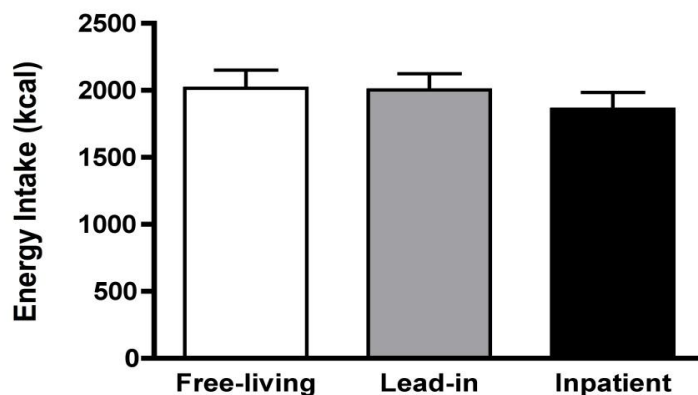
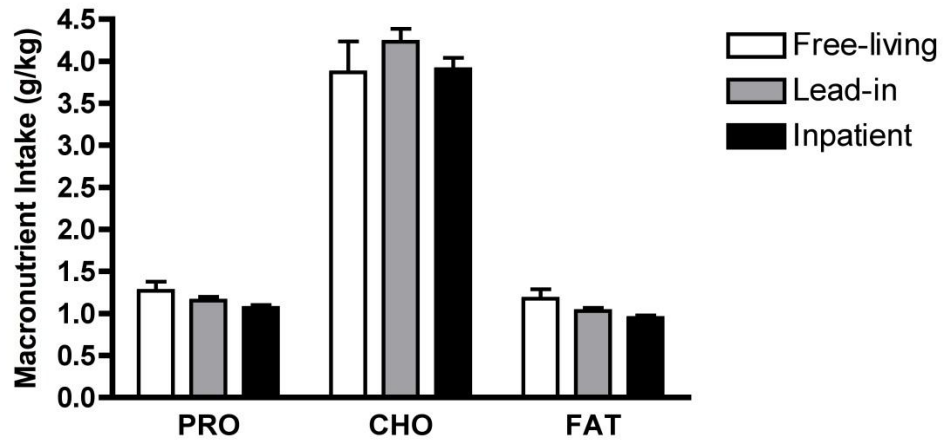


Figure 4.2 Macronutrient intakes for the free-living, lead-in, and inpatient diets



Energy expenditure

PAL and Non-exercise PAL levels were not significantly different between the CHO and PRO+CHO trials (Figure 4.3 and Figure 4.4, respectively).

Figure 4.3 PAL during inpatient CHO and PRO+CHO trials.

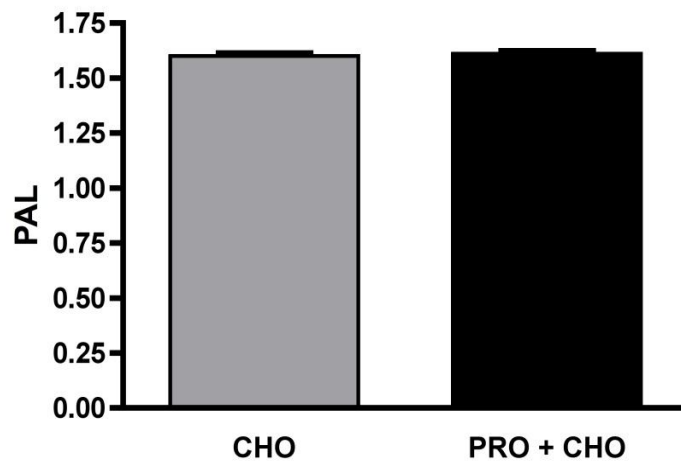
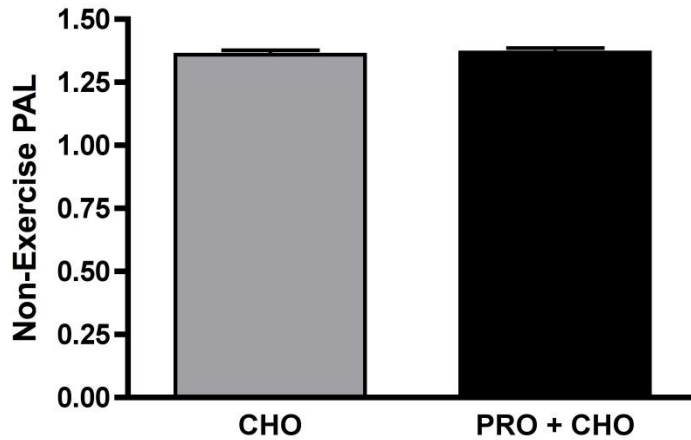


Figure 4.4 Non-exercise PAL during inpatient CHO and PRO+CHO trials



#### Energy balance

Subjects were in energy balance during the lead-in diet. During the inpatient period, subjects were in negative energy balance with a mean daily negative energy balance of  $-13.96 \pm 1.82\%$  for the CHO trial, and  $-14.01 \pm 1.81\%$  for PRO+CHO trial (Figure 4.5A). Energy balance was not significantly different between the CHO and PRO+CHO trials ( $p=0.29$ ). On average, subjects were in  $-284.37 \pm 34.68$  kcal during the CHO trial and  $-291.14 \pm 48.42$  kcal during the PRO+CHO trial (Figure 4.5B). Additionally, energy balance between Days 1-3 (trial 1) and Days 4-6 (trial 2) was not significantly different ( $p=0.10$ ) (Figure 4.6).

Figure 4.5 Energy balance during CHO and PRO+CHO trials, expressed as % and kcals

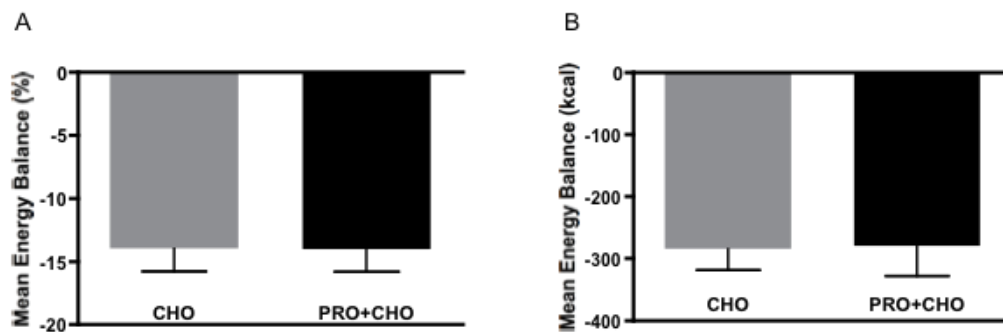
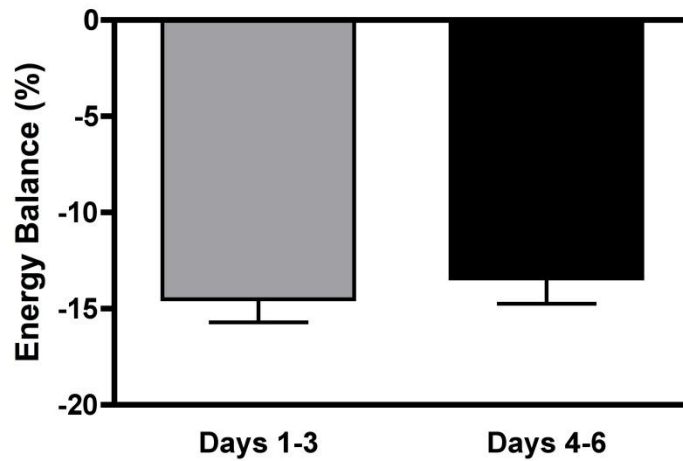


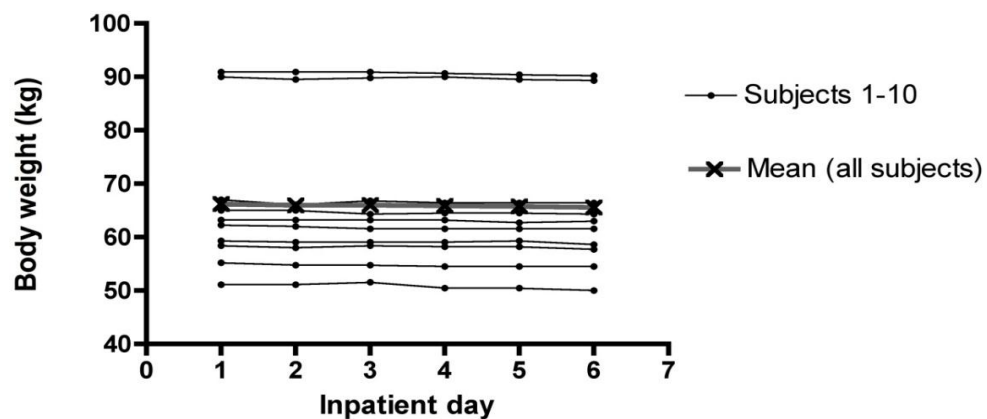
Figure 4.6 Mean inpatient energy balance on Days 1-3 and Days 4-6



#### Weight and body composition

Body weight did not significantly change from pre ( $67.2 \pm 4.4$  kg) to post ( $66.7 \pm 4.3$  kg) ( $p=0.051$ ) although there was a strong trend. Additionally, body fat percentage did not significantly change from pre ( $33.2 \pm 1.7\%$ ) to post ( $34.0 \pm 2.2\%$ ) ( $p=0.18$ ), nor did fat free mass (pre:  $44.0 \pm 3.5$  kg and post:  $43.5 \pm 3.9$  kg) ( $p=0.24$ ). The tracking of individual daily body weight during the inpatient period is shown in Figure 4.7. Mean body weight decreased slightly from inpatient Day 1 ( $66.21 \pm 4.29$ kg) to Day 6 ( $65.60 \pm 4.31$ kg), indicating subjects were in negative energy balance.

Figure 4.7 Daily inpatient bodyweight for individual subjects (including group mean)





### Nitrogen Balance (NBAL)

Three-day average nitrogen balance was not significantly different between the CHO and the PRO+CHO trials ( $p=0.28$ ) (Figure 4.8). The mean nitrogen balance for the CHO trial was  $-0.070 \pm 1.644$  g nitrogen (N), and  $0.097 \pm 1.664$  g N in the PRO+CHO trial. Mean nitrogen balance data for the CHO and PRO+CHO trials for individual subjects is presented in Figure 4.7. Additionally, there was not a significant difference in nitrogen balance between Days 1-3 and Days 4-6 ( $p=0.245$ ) (Figure 4.9). Even though subjects were in negative energy balance, nitrogen balance was not different from zero.

Figure 4.8 3-day mean NBAL during CHO and PRO+CHO trials

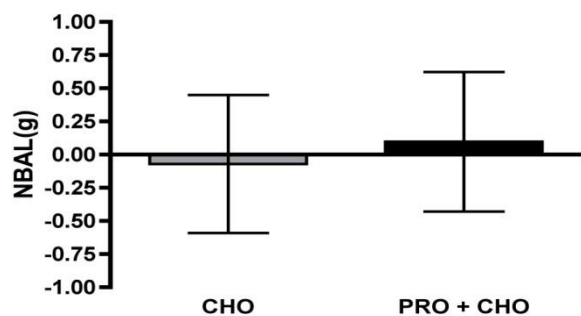


Figure 4.9 Mean NBAL of individual subjects and group mean during CHO and PRO+CHO trials

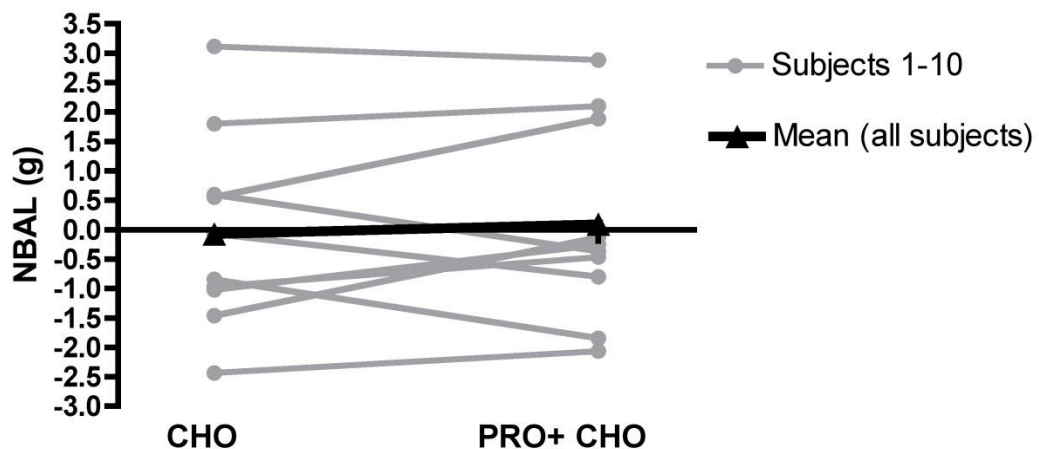
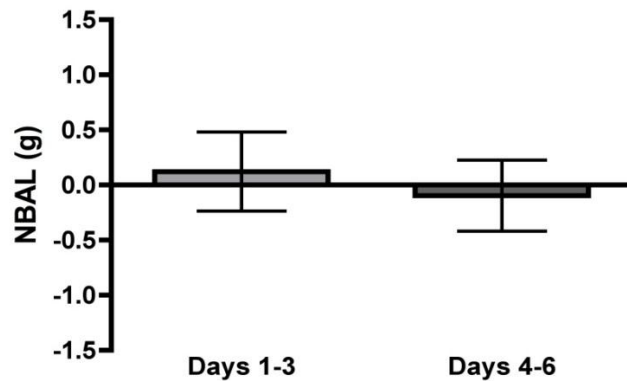


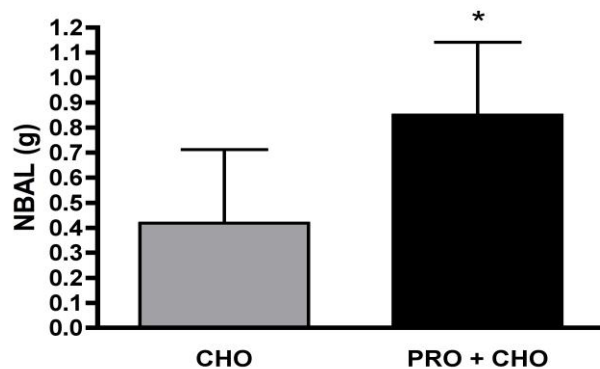
Figure 4.10 Mean NBAL during Days 1-3 and Days 4-6



**Combined data for negative, even, and positive energy balance studies**

Data from all three energy cohorts (negative, even, positive) of the study were combined. When the data from all three energy cohorts were combined, nitrogen balance was significantly greater in the PRO+CHO trial ( $0.851 \pm 0.291$  g N) than the CHO trial ( $0.419 \pm 0.294$  g N) ( $p=0.01$ ) (Figure 4.11).

Figure 4.11 Mean NBAL in CHO and PRO+CHO trials in all studies combined (negative, even, and positive energy balance)



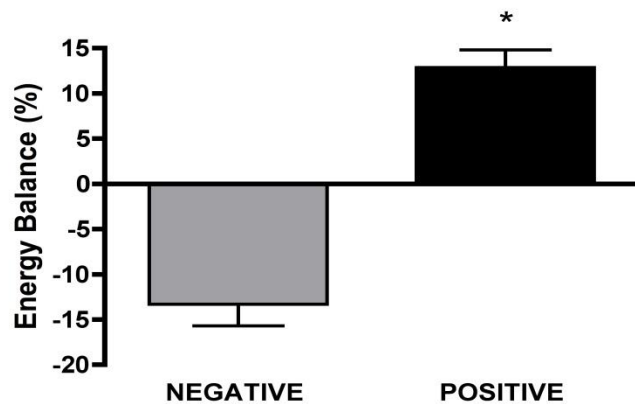
Data from all three energy cohorts were then re-stratified in order to make further comparisons between nitrogen balance and energy balance. Six-day mean energy balance was used to re-stratify the groups. Less than 0.0% energy balance was considered negative, and greater than

0.0% energy balance was considered positive. Overall, there were nine subjects in the negative energy balance group and fourteen subjects within the positive energy balance group.

#### Re-stratified Energy Balance

After restratification, the resulting energy balance was significantly different between the negative and positive energy balance groups ( $p < 0.0001$ ). Mean negative energy balance was  $-13.39 \pm 2.41\%$ , and mean positive energy balance was  $12.85 \pm 1.96\%$  (Figure 4.12).

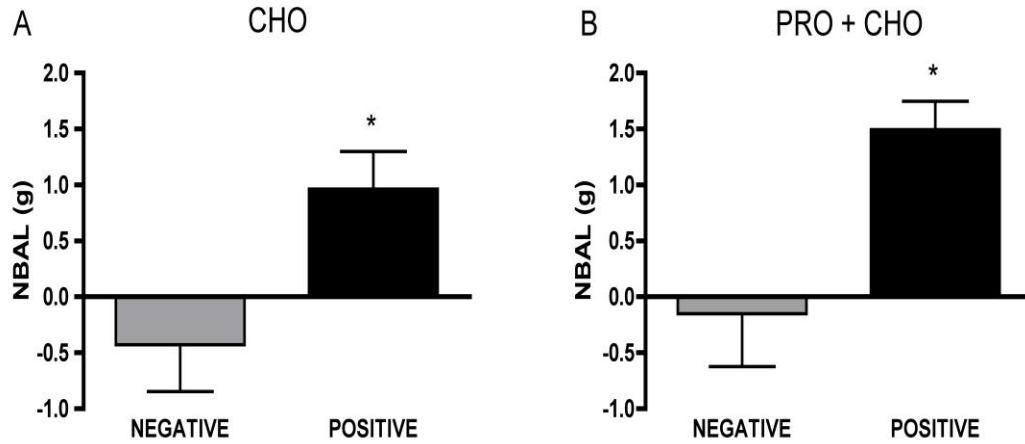
Figure 4.12 Mean energy balance in re-stratified negative and positive energy balance groups



#### Re-stratified Nitrogen Balance

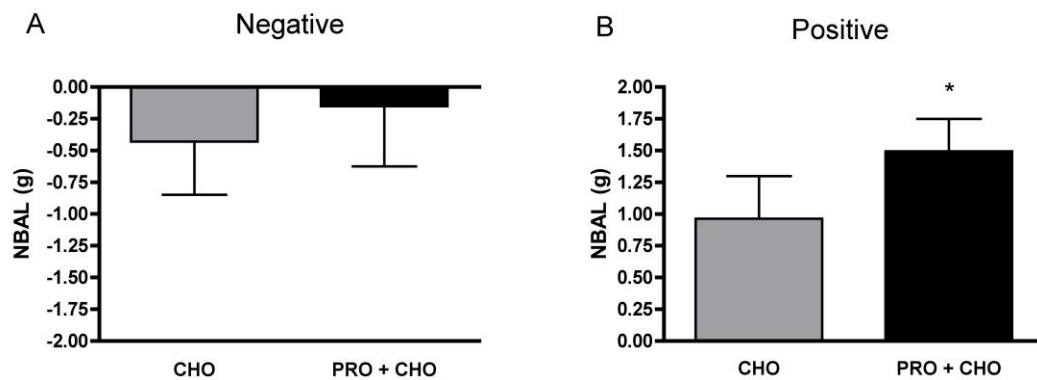
The mean difference (PRO+CHO trial minus CHO trial) in nitrogen balance between the CHO and PRO+CHO trial was not significant ( $p = 0.244$ ). However, the mean difference in nitrogen balance was significantly different for both the CHO trial ( $p = 0.009$ ), and the PRO+CHO trial ( $p = 0.002$ ). The mean nitrogen balance values for the CHO trial were  $-0.429 \pm 0.418$ g nitrogen for the negative group, and  $0.964 \pm 0.336$ g nitrogen for the positive group (Figure 4.13). The mean nitrogen balance values for the PRO+CHO trial were  $-0.151 \pm 0.473$ g nitrogen for the negative group, and  $1.49 \pm 0.256$ g nitrogen for the positive group (Figure 4.13)

Figure 4.13 Mean NBAL in re-stratified negative and positive groups during CHO trial ( $p=0.009$ ) and PRO+CHO trial ( $p=0.002$ )



There was not a significant difference in mean nitrogen balance between the CHO and PRO+CHO trial in the re-stratified negative energy balance group ( $p=0.178$ )(Figure 4.14A). However, there is a significant difference between the CHO and PRO+CHO trial in the re-stratified positive energy balance group ( $0.964 \pm 0.336$ g nitrogen for the CHO trial, and  $1.494 \pm 0.256$ g nitrogen for the PRO+CHO trial)( $p=0.016$ ) as depicted in Figure 4.14B.

Figure 4.14 Mean nitrogen balance in CHO and PRO+CHO trials for re-stratified negative energy balance and positive energy balance groups

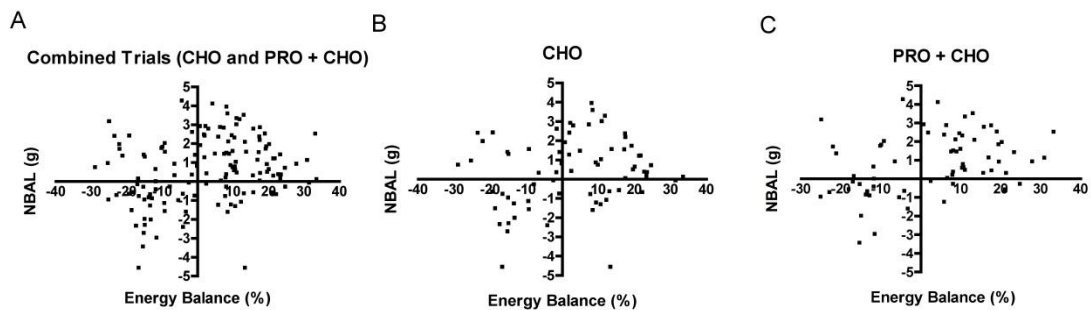


### Re-stratified Energy balance and Nitrogen Balance

Daily energy balance was significantly correlated with daily nitrogen balance ( $R^2=0.065$ ,  $p=0.003$ ) (Figure 4.15). Within the PRO+CHO trial, daily energy balance is significantly correlated with daily nitrogen balance ( $R^2=0.115$ ,  $p=0.004$ ) (Figure 4.15). However, within the CHO trial, energy balance was not significantly correlated to nitrogen balance ( $R^2=0.035$ ,  $p=0.127$ ) (Figure 4.15).

Figure 4.15 Correlation between daily energy balance and daily NBAL in Combined Trials

( $p=0.003$ ), CHO trial ( $p=0.127$ ), and PRO + CHO trial ( $p=0.004$ )



### Re-stratified Protein intake and Nitrogen Balance

Daily protein intake (g/kg bw) for all subjects within the three cohorts was not significantly correlated with daily nitrogen balance ( $R^2=0.003$ ,  $p=0.490$ ) (Figure 4.16). However, when the data is further partitioned by sex, daily protein intake (g/kg bw) was significantly correlated with nitrogen balance for both males ( $R^2=0.328$ ,  $p<0.0001$ ) (figure 4.16) and females ( $R^2=0.042$ ,  $p=0.028$ ) (Figure 4.16). For both males and females, nitrogen balance was then correlated to daily protein intake (g/kg bw) for the CHO and PRO+CHO trials. In females, daily protein intake (g/kg bw) was not significantly correlated to daily nitrogen balance in either the CHO ( $R^2=0.051$ ,  $p=0.091$ ), or the PRO+CHO trial ( $R^2=0.035$ ,  $p=0.162$ ). However, in males, daily protein intake (g/kg bw) was significantly correlated to daily nitrogen balance in both the CHO ( $R^2=0.252$ ,  $p=0.021$ ) (Figure 4.17), and the PRO+CHO trial ( $R^2=0.435$ ,  $p=0.001$ ) (Figure 4.17).

Figure 4.16 Correlation between daily protein intake (g/kg bw) and daily NBAL for all subjects ( $p=0.490$ ), females ( $p=0.028$ ), and males ( $p<0.0001$ )

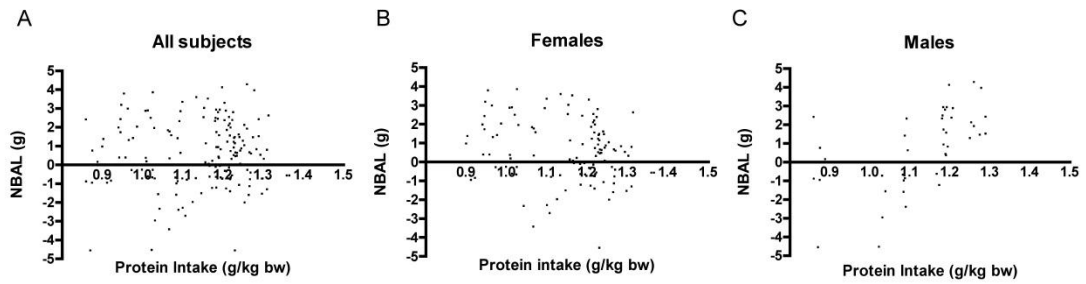
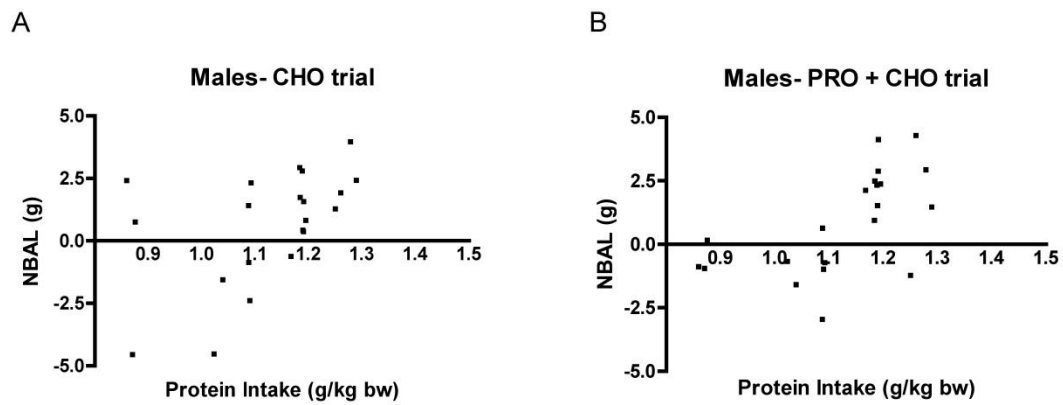


Figure 4.17 Correlation between daily protein intake (g/kg bw) and daily NBAL for men during CHO ( $p=0.021$ ), and PRO+CHO trials ( $p=0.001$ )



## DISCUSSION

The current study investigated the timing of protein consumption in relation to a bout of moderate aerobic exercise on nitrogen balance. Older individuals completed two 3-day trials with only the timing of protein consumption during the day differing between the two conditions. Contrary to our initial hypothesis, nitrogen balance did not significantly differ in older individuals in negative energy balance when protein was consumed immediately after moderate aerobic exercise rather than earlier in the day. Previously, our lab showed that nitrogen balance was significantly greater in older individuals in energy balance when protein was consumed immediately after moderate aerobic exercise compared to earlier in the day. Together these results indicate that energy balance is an important determinant of the anabolic effect of protein feeding. Moreover, older individuals were able to maintain nitrogen balance by timing nutrient intake immediately after aerobic exercise, despite being in negative energy balance. To our knowledge, our study was the first to investigate the combined effects of varying energy balance and the timing of protein intake on nitrogen balance in older individuals.

Nitrogen balance is closely related to energy balance such that nitrogen balance is better maintained when caloric intake is adequate, as a negative nitrogen balance results when energy intake is reduced (Todd, Butterfield et al. 1984). During caloric restriction, protein breakdown increases the availability of amino acids that are oxidized as energy and thus limits the availability of amino acids for protein synthesis. When caloric intake is inadequate, aerobic exercise can increase nitrogen balance (Todd, Butterfield et al. 1984). Consistent with this, in

the current study older individuals were able to maintain nitrogen balance for both the CHO and PRO+CHO trials despite being in approximately 14% negative energy balance. Since loss of lean body mass occurs when individuals are in negative nitrogen balance (Friedlander, Braun et al. 2005), maintaining nitrogen balance while in negative energy balance could reduce the loss of lean body mass and thus attenuate the progression of sarcopenia and wasting.

The daily exercise completed by participants during the inpatient stay was one hour of moderate intensity (55%  $VO_{2max}$ ) cycling. The exercise intensity and duration was well-tolerated by all subjects, and the inpatient PAL was consistent with older populations (Pannemans and Westerterp 1995). Exercise at 55% of  $VO_{2max}$  provides an effective anabolic stimulus, as aerobic exercise at only 40% of  $VO_{2max}$  can increase muscle protein synthesis (Sheffield-Moore, Yeckel et al. 2004). Additionally, exercise at 55% of  $VO_{2max}$  simulates a brisk walking pace and since older individuals most commonly choose walking as exercise (McPhillips, Pellettera et al. 1989), the proposed exercise intervention is practical for an older population.

All study diets followed USDA dietary recommendations for the percentage of total kilocalories for each macronutrient (45-65% carbohydrate, 20-35% fat, and 10-35% protein) (UnitedStates.Dept.Agriculture 2005) and were: 55% carbohydrate, 30% fat, and 15% protein. Inpatient protein intake was 1.1 g/kg bw, which exceeds the 0.8 g/kg bw recommendation for older individuals; however, it is still representative of a typical protein intake for an older population (Millward and Roberts 1996). Mean self-reported habitual protein intake was  $1.3 \pm 0.1$  g/kg bw, thus the lead-in diet allowed study participants to adjust to the new level of protein intake. Furthermore, mean nitrogen balance did not differ between inpatient Days 1-3 and Days 4-6, which indicates that participants were habituated to the inpatient protein intake.

Older individuals have a blunted muscle protein synthesis response to anabolic signals, compared to younger individuals, as the anabolic response of skeletal muscle proteins to



essential amino acids is reduced in the elderly (Volpi, Kobayashi et al. 2003). However, increasing the proportion of leucine allows for optimal stimulation of muscle protein synthesis in older individuals (Katsanos, Kobayashi et al. 2006). Chocolate milk was chosen for this study as the protein source because milk contains adequate leucine, which enhances protein synthesis through the mTOR pathway (Anthony, Anthony et al. 2000). Moreover, milk consists of both whey and casein proteins (Jenness 1979). Whey protein results in an acute increase in protein synthesis (Boirie, Dangin et al. 1997), whereas casein protein results in a minor increase in protein synthesis and a significant decrease in protein breakdown (Boirie, Dangin et al. 1997). Thus, milk can enhance protein accretion by increasing protein synthesis and down-regulating protein breakdown. In this study, the chocolate milk beverage consisted of 11.3 g protein within the skim milk, supplemented with 4 g of whey protein. Overall, the chocolate milk beverage contained 15.3 g protein including 6.8 g essential amino acids with 1.7 g leucine. The chocolate milk also consisted of 43.6 g carbohydrate. Consumption of carbohydrate combined with protein after aerobic exercise increases whole body net protein balance and muscle FSR more than an isocaloric amount of carbohydrate alone (Howarth, Moreau et al. 2009). The consumption of chocolate milk as a post-exercise beverage would be practical for an older population as it is an easily attainable protein source.

#### Combined data from all three energy cohorts

Data from all three energy cohorts (negative, even, positive) of the study were combined in order to further analyze the relationship between energy balance and the timing of protein intake on nitrogen balance. Combining the data from all three energy cohorts is appropriate since energy balance typically varies from day to day. When the data from the three cohorts were combined mean nitrogen balance was significantly greater when protein and carbohydrates are consumed immediately after exercise rather than earlier in the day.

Therefore, consuming protein and carbohydrate immediately after exercise is a valid approach to increase nitrogen balance in older individuals.

The subjects were also stratified into a negative or positive energy balance group in order to analyze nitrogen balance for both the PRO+CHO and CHO trials. Six-day mean energy balance was used to re-stratify the groups. Any 6-day mean energy balance that was less than 0.0% was placed into the negative energy balance group and any 6-day mean nitrogen balance that was greater than 0.0% was placed into the positive energy balance group. Overall, the new negative energy balance group contained nine subjects and the positive energy balance group contained fourteen subjects. Three subjects were excluded from the re-stratification because the accelerometer malfunctioned during their inpatient stay and therefore six-day mean energy balance was not available for the three subjects.

#### *Energy Balance and Nitrogen Balance*

Within the re-stratified negative energy balance group, there was not a significant difference in nitrogen balance between the PRO+CHO and CHO trials. Although the lack of significance between the PRO+CHO and CHO trials is consistent with the results from the negative energy balance cohort, these results differ from previous research by Roy et al. (Roy, Luttmer et al. 2002). In young female athletes in exercise-induced negative energy balance, timing nutrient intake immediately after exercise, rather than earlier in the day, resulted in greater nitrogen balance with a strong trend toward significance ( $p=0.06$ ) (Roy, Luttmer et al. 2002). Within the re-stratified positive energy balance group, nitrogen balance was significantly different between the PRO+CHO and CHO trials. Moreover, across the re-stratified energy groups, there was a significant correlation between daily energy balance and nitrogen balance. Taken together, our results indicate that timing protein intake immediately after aerobic

exercise has a greater influence on nitrogen balance when in positive energy balance rather than negative energy balance.

Within the re-stratified energy balance groups, mean nitrogen balance was significantly different between the negative and positive energy balance groups for both the CHO trial and the PRO+CHO trial. The increase in nitrogen balance was more prominent in the PRO+CHO trial than the CHO trial, which is consistent with previous research (Howarth, Moreau et al. 2009). Howarth et al. found that consuming protein with carbohydrate after aerobic exercise increases whole body net protein balance more than an isocaloric amount of carbohydrate alone (Howarth, Moreau et al. 2009). Thus, consuming protein with carbohydrate after aerobic exercise increases nitrogen balance more than consuming carbohydrate alone.

Across the re-stratified energy groups, there was a significant correlation between energy balance and nitrogen balance when protein and carbohydrate was consumed post-exercise (PRO+CHO group). Within the CHO group, there was not a significant correlation between daily energy balance and daily nitrogen balance. These results indicate that energy balance is more predictive of nitrogen balance when protein with carbohydrate is consumed immediately post-exercise compared to an isocaloric amount of only carbohydrate.

#### Protein Intake and Nitrogen Balance

Daily protein intake (g/kg bw) was not significantly correlated with daily nitrogen balance. However, when the subjects were split by sex, daily protein intake was significantly correlated with nitrogen balance in males ( $R^2=0.3279$ ,  $p<0.0001$ ). Similarly, in females daily protein intake was significantly correlated with nitrogen balance ( $R^2=0.04219$ ,  $p=0.0284$ ). These results indicate that total daily protein intake (g/kg bw) is predictive of nitrogen balance when separated by sex. Moreover, the male and female groups were further divided by PRO+CHO and CHO trials. We found that in males, daily total protein intake is significantly correlated with

nitrogen balance in both the PRO+CHO ( $R^2=0.4346$ ,  $p=0.0012$ ) and CHO trials ( $R^2=0.2515$ ,  $p=0.0205$ ). In females, total protein intake was not significantly correlated with nitrogen balance for either PRO+CHO or CHO trials. These results suggest that in older males, protein intake is more predictive of nitrogen balance when protein with carbohydrate is consumed post-exercise, rather than an isocaloric amount of only carbohydrate. To our knowledge, the sex related differences between protein intake and protein timing on nitrogen balance is a novel finding. The sex related differences on nitrogen balance may be due to differences in the rates of protein synthesis during basal conditions between older men and older women (Smith, Atherton et al. 2008).

### Limitations

Use of the nitrogen balance technique may result in inflated nitrogen retention values (Kopple 1987). Nitrogen intake can be overestimated if any food is not completely consumed. However, participants were instructed to consume all foods and consumption of the inpatient meals was closely monitored to ensure that all food and beverages were consumed. Furthermore, nitrogen intake is estimated based on total protein intake, with 6.25 grams of protein containing 1 gram of nitrogen. However, 6.25 grams of protein do not always contain 1 gram of nitrogen, as 6.25 is an average based on protein quality. Inadequate collection of any excreted nitrogen can also contribute to overestimated nitrogen retention. Collecting miscellaneous nitrogen losses can be quite challenging and therefore relies on estimates. Thus, variability between individuals could contribute to overestimated nitrogen balance. However, the same foods were consumed during both trials, and any measurement error would occur during both conditions to the same extent. Therefore, if nitrogen balance was overestimated, the difference in nitrogen balance between the two conditions would still remain constant.

Despite limitations, the nitrogen balance technique can be a useful technique for studying whole body protein turnover in aging populations. The contribution of muscle protein to whole body protein metabolism is significantly reduced in the elderly. Consequently, the contribution of non skeletal muscle protein, especially visceral tissue whose rates of protein turnover are known to be more rapid, is proportionally greater with aging (Morais, Chevalier et al. 2006). Thus, the nitrogen balance technique with its noninvasive nature makes it a relatively easy and appropriate method to determine long term changes in whole body protein turnover in response to exercise and nutrition interventions in older individuals.

An error in the nutritional information for the carbohydrate beverage during the energy balance cohort of the study resulted in an additional 159 grams of carbohydrate in the CHO beverage. Therefore, subjects in the even energy balance cohort received an additional 159 calories post-exercise in the CHO trial compared to the PRO+CHO trial. Since the effect of carbohydrate on protein synthesis depends on the amount consumed (Miller, Tipton et al. 2003), nitrogen balance for the ten subjects in the CHO condition may have been more positive than if the CHO beverage was isocaloric with the PRO+CHO beverage or with the other CHO beverage.

#### Conclusions and future directions

In summary, the current study investigated whether timing protein intake after moderate aerobic exercise can improve nitrogen balance in older individuals in negative energy balance. We hypothesized that nitrogen balance would increase when protein was consumed post-exercise. However, contrary to our initial hypothesis, nitrogen balance did not significantly differ when protein was consumed immediately after exercise rather than earlier in the day. Previously, our lab showed that nitrogen balance was significantly greater in older individuals in energy balance when protein was consumed immediately after aerobic exercise compared to

earlier in the day. Together these results indicate that energy balance is an important determinant of the anabolic effect of protein feeding and therefore must be considered when using the NBAL method.

We found significant differences with protein intake on nitrogen balance between sexes; therefore, future studies should investigate whether nitrogen balance differs between males and females under various nutritional and exercise interventions. Additionally, future studies should explore whether increases in nitrogen balance observed within this study can be maintained in the long term.

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## APPENDIX I



Consent Form Approval

Date: \_\_\_\_\_

Valid For Use Through: \_\_\_\_\_

**SUBJECT CONSENT FORM**

**COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD**

Protocol #08-0640

And CSU IRB

Protocol # 08-187H

“Consumption of milk after physical activity - rethinking protein recommendations in older individuals”

PRINCIPAL INVESTIGATOR: Edward L. Melanson, Ph. D. and Benjamin F Miller, PhD

Version #2

Last Updated: April 16, 2010

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You are being asked to be in a research study. This form provides you with information about the study. A member of the research team will describe this study to you and answer all of your questions. Please read the information below and ask questions about anything you don't understand before deciding whether or not to take part.

**Why is this study being done?**

This study plans to learn more about how to prevent muscles from wasting with the aging process. We would like to use a simple strategy using exercise and

nutrition. What this study seeks to determine is when you have protein can be just as important as how much protein you receive. In the two study periods what you eat will be the same, but when you eat it will be different.

You are being asked to be in this research study because you are a healthy individual aged 55-75.

**Other people in this study**

Up to 40 people from your area will participate in the study.

**What happens if I join this study?**

If you join the study, you will first complete a physical screening including a screening for heart disease at Colorado State University. We will ask you to answer some questions about your past and current participation in exercise. Your body weight, height, and body composition will be measured by laying in a bed and being scanned. Your gender and date of birth will also be

recorded. A 3 tsp sample of blood will be taken for screening purposes. Before undergoing the sampling procedures, you will be asked a few questions relating to your present state of health, current medication and past medical history. This is to exclude the presence of any condition or medication that might prolong your bleeding time or make the blood sampling unsafe for you. This visit will take approximately 30 minutes.

You will also undergo cardiac screening in the presence of a cardiologist. This will involve the placement of ten collecting electrodes on your chest that will be connected to an electrocardiogram (ECG). You will then be asked to walk on a treadmill, slowly at first and progressively faster until the cardiologist asks you to stop. This test will take approximately 45 minutes. If anything adverse is found in any of the medical screening, you will be advised.

In the seven-day period leading up to the study, we will provide you with all of your food from the Department of Food Science and Human Nutrition at CSU. The food will be normal food tailored to your diet, but we ask that you only consume this food.

For the study, you will report to the general clinical research center (GCRC) at the Colorado Health Sciences Center the night before the start of the study and sleep over night. You will then remain in the GCRC for six days. During this stay you will be asked to exercise once per day for one hour on a bike. The exercise intensity will be slightly more than that required to complete a brisk walk. During the entire stay you will also be fed a controlled diet that will be slightly less (approximately 200 kcal) than your normal calorie and protein intake and you will wear a monitor to measure the amount of activity. You will receive a protein supplement either in the morning or after your exercise bout and when you receive the protein supplement will switch half way through the stay. Throughout the study all your urine will be collected for analysis. In addition, we will collect a small blood sample (2 tsp) at three different times. For a 24 hr period at the beginning of the study and at the end of the study, you will reside in a room that will measure the rate your body is using energy. This room contains everything you will need for normal living, but is specially designed for our measurements. After you exit the special room on the last day, you will receive one more body scan for body composition. In total your commitment to the study is a half-day for screening, seven days with normal living and food provision, and a six-day (seven-night) stay at the GCRC.

#### **What are the possible discomforts or risks?**

In this study we will need to get a total of about 9 teaspoons of blood from you. We will get blood by putting a needle into one of your veins and letting the blood flow into a glass tube. You may feel some pain when the needle goes into your vein. A day or two later, you may have a small bruise where the needle went under the skin. If you have not participated in a regular exercise program before, you may experience some discomfort with the exercise bout including muscle soreness or labored breathing. You may experience discomfort (boredom) associated with a prolonged stay in our facility and special room. However, all efforts will be made to ensure your comfort including access to television, internet, and daily exercise.

Other possible risks include a small risk (less than 1 in 10 000) of death due to a cardiac event during exercise screening. There are also risks of fatigue and muscle strains. You may experience temporary breathlessness or dizziness towards the end of the test. These feelings

are transient and pass once the test is finished. During your daily exercise there is also a risk of cardiac complications but in individuals with good cardiac health this risk is extremely low (1 in 1 000 000).

As part of this study we will perform two DEXA scans of your body. DEXA is a way of looking inside the body by using X-rays. X-rays are a type of radiation. Your natural environment has some radiation in it. This DEXA will give you about the same amount of radiation that you would get from your environment in four days.

This study may include risks that are unknown at this time

**What are the possible benefits of the study?**

This study is designed for the researcher to learn more about potential non-pharmaceutical treatments for the prevention of muscle wasting with aging. We want to incorporate easy-to-follow strategies for improved muscular health. You will receive a medical and cardiac screen and will obtain information on your body composition.

This study is not designed to treat any illness or to improve your health. Also, there may be risks, as discussed in the section describing the discomforts or risks.

**Who is paying for this study?**

This research is being funded by the Colorado Agricultural Experiment Station with the mission of increasing the quality of foods in Colorado.

**Will I be paid for being in the study?**

You will not be paid for the screening tests or travel expenditures, but you will be paid \$300 for completion of the GCRC stay. If you leave the study early, or if we have to take you out of the study, you will be paid \$40 for each overnight stay.

It is important to know that payments for participation in a study are taxable income.

**Will I have to pay for anything?**

It will not cost you anything to be in the study.

**Is my participation voluntary?**

Taking part in this study is voluntary. You have the right to choose not to take part in this study. If you choose to take part, you have the right to stop at any time. If you refuse or decide to withdraw later, you will not lose any benefits or rights to which you are entitled.

**Can I be removed from this study?**

The study nurse, Rebecca Benson PA/RN, or her supervising physician, Robert Eckel, MD, may decide to stop your participation without your permission if the study nurse/doctor thinks that being in the study may cause you harm, or for any other reason.

**What happens if I am injured or hurt during the study?**

You should inform your care provider(s) if you decide to participate in this research study. If you have questions about injury related to the research, you may call the study coordinator, Edward Melanson, Ph.D. at (303) 724-0935 and/or your private physician. Edward Melanson, Ph.D. should be informed about any injury you experience while you take part in this study. If you are hurt by this research, we will give you medical care if you want it, but you will have to pay for the care that is needed.

**Who do I call if I have questions?**

The researchers carrying out this study are Dr. Ed Melanson and Dr. Benjamin Miller. You may ask any questions you have now. If you have questions later, you may call Dr. Melanson at 303-724-0935 or Dr. Miller at 970-491-3291. You will be given a copy of this form to keep.

You may have questions about your rights as someone in this study. You can call Dr. Melanson at 303-724-0935 or Dr. Miller at 970-491-3291 with questions. You can also call the Colorado Multiple Institutional Review Board (COMIRB). You can call them at 303-724-1055.

The main person to talk to if you have questions about this study is Dr. Melanson at 303-724-0935 or Dr. Miller at 970-491-3291. You can also talk to a Subject Advocate at the General Clinical Research Center (GCRC)/ the Clinical Translation Research Center (CTRC). The phone number there is 720-848-6662.

**Who will see my research information?**

We will do our best to keep your research records private. But there are some people and agencies who will be allowed to see them. These include:

- Federal offices such as the Food and Drug Administration (FDA) that protect research subjects like you.
- People at the Colorado Multiple Institutional Review Board (COMIRB)
- The study doctor and his/her team of researchers.
- Officials at Colorado State University or the Colorado Health Sciences Center who are in charge of making sure that we follow all of the rules for research

We might talk about this research study at meetings. We might also print the results of this research study in relevant journals. But we will always keep the names of the research subjects, like you, private.

We will ask you to sign a different form that talks about who can see your research records. That form is called a HIPAA form. It will mention companies and universities who will see your research records.

You have the right to request access to your personal health information from the Investigator. [To ensure proper evaluation of test results, your access to these study results may not be allowed until after the study has been completed – if applicable].

This HIPAA authorization does not expire. However, you may withdraw this authorization for use and disclosure of your personal health information by providing written request to the Investigator. If you withdraw this authorization, the Institution, the Investigator, the research staff, and the research Sponsor will no longer be able to use or disclose your personal health information from this study, except so far as that they have already relied on this information to conduct the study.

**Agreement to be in this study**

I have read this paper about the study or it was read to me. I understand the possible risks and benefits of this study. I know that being in this study is voluntary. I choose to be in this study: I will get a copy of this consent form.

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Print Name: \_\_\_\_\_

Consent form explained by: \_\_\_\_\_ Date: \_\_\_\_\_

Print Name: \_\_\_\_\_

Investigator: \_\_\_\_\_ Date: \_\_\_\_\_

## APPENDIX II

Authorization To Use or Release Health Information About Me For Research Purposes <i>Authorization B: Enrollment into Research</i>	Study Title: Consumption of milk after physical activity - rethinking protein recommendations in older individuals   COMIRB Number: 08-0460
I _____ (Subject's Full Name) <b>authorize</b>  _____ (PI or Physician Name) <b>and</b> <b>staff members of</b>  _____ (Facility Name) <b>working for him/her to use the following health information about me for research:</b> (Please check the appropriate boxes. NOTE: If a category is checked "yes" and a line follows the category, you MUST describe the type of the procedures done.)	

<b>No Yes</b>  <input type="checkbox"/> <input type="checkbox"/> Name and/or phone number  <input type="checkbox"/> <input type="checkbox"/> Demographic information (age, sex, ethnicity, address, etc.)  <input type="checkbox"/> <input type="checkbox"/> Diagnosis(es)  <input type="checkbox"/> <input type="checkbox"/> History and/or Physical  <input type="checkbox"/> <input type="checkbox"/> Laboratory or Tissue Studies: _____ <input type="checkbox"/> <input type="checkbox"/> Radiology Studies: _____ <input type="checkbox"/> <input type="checkbox"/> Testing for or Infection with Human Immunodeficiency Virus (HIV) (or results) <input type="checkbox"/> <input type="checkbox"/> Procedure results: _____
---





**The PI (or staff acting on behalf of the PI) will also make the following health information about me available to:** (check all that apply and **describe the type of the procedures** done where applicable)

Recipient (name of person or group)

**No Yes**

☐ ☐ All Research Data Collected in this Study (if you check this box Yes, no other boxes need to be checked in this section)

☐ ☐ All Research Data Collected in this Study except for name, phone number, and/or address (if you check this box Yes, no other boxes need to be checked in this section)

☐ ☐ Name and phone number

☐ ☐ Demographic information (age, sex, ethnicity, address, etc.)

☐ ☐ Diagnosis(es)

☐ ☐ History and Physical

☐ ☐ Laboratory or Tissue Studies: \_\_\_\_\_

☐ ☐ Radiology Studies: \_\_\_\_\_

☐ ☐ Testing for or Infection with Human Immunodeficiency Virus (HIV) (or results)

☐ ☐ Procedure results: \_\_\_\_\_

☐ ☐ Psychological tests: \_\_\_\_\_

☐ ☐ Questionnaire/Survey: \_\_\_\_\_

☐ ☐ Research Visit records

☐ ☐ Portions of previous Medical Records that are relevant to this study

☐ ☐ Billing/Charges

☐ ☐ Drug Abuse

☐ ☐ Alcoholism or Alcohol

☐ ☐ Sickle Cell Anemia

☐ ☐ Other (Specify): \_\_\_\_\_

**For the Specific Purpose of**

- ☐ Evaluation of this research project
- ☐ Evaluation of laboratory/tissue samples
- ☐ Data management
- ☐ Data analysis
- ☐ Other\*: \_\_\_\_\_

*\*Cannot say "for any and all research", "for any purpose", etc.*

*For additional Recipients, copy this page as needed.*

I give my authorization knowing that:

I do not have to sign this authorization. But if I do not sign it the researcher has the right to not let me be in the research study.

I can cancel this authorization any time.

1. I have to cancel it in writing.
2. If I cancel it, the researchers and the people the information was given to will still be able to use it because I had given them my permission, but they won't get any more

information about me.

3. If I cancel my authorization, I may no longer be able to be in the study.
4. I can read the Notice of Privacy Practices at the facility where the research is being conducted to find out how to cancel my authorization.  
The records given out to other people may be given out by them and might no longer be protected.
6. I will be given a copy of this form after I have signed and dated it.

This authorization will expire on: \_\_\_\_\_ (Date) OR

☐ The end of the research study

☐ Will not expire

☐ \_\_\_\_\_

(Describe dates or circumstances under which the authorization will expire.)

\_\_\_\_\_

ADDITIONAL INFORMATION: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

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Subject's Signature	Date
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Signature of Legal Representative (If applicable)	Date
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Name of Legal Representative (please print)

---

Description of Legal Authority to Act on Behalf of Patient

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Site: Colorado State University, Dr. Benjamin Miller

I give my authorization knowing that:

I do not have to sign this authorization. But if I do not sign it the researcher has the right to not let me be in the research study.

I can cancel this authorization any time.

9. I have to cancel it in writing.

10. If I cancel it, the researchers and the people the information was given to will still be able to use it because I had given them my permission, but they won't get any more information about me.

11. If I cancel my authorization, I may no longer be able to be in the study.

12. I can read the Notice of Privacy Practices at the facility where the research is being conducted to find out how to cancel my authorization.

The records given out to other people may be given out by them and might no longer be protected.

14. I will be given a copy of this form after I have signed and dated it.

This authorization will expire on: \_\_\_\_\_ (Date) OR

☐ The end of the research study

☐ Will not expire

☐ \_\_\_\_\_

(Describe dates or circumstances under which the authorization will expire.)

ADDITIONAL INFORMATION: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Subject's Signature

Date

\_\_\_\_\_

Signature of Legal Representative (If applicable)	Date
<hr/>	
Name of Legal Representative (please print)	
<hr/>	
Description of Legal Authority to Act on Behalf of Patient	

### APPENDIX III

DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

**MEDICAL AND EXERCISE HISTORY**

NAME \_\_\_\_\_ GENDER \_\_\_\_\_ DATE \_\_\_\_\_

BIRTHDATE \_\_\_\_\_ AGE \_\_\_\_\_ HEIGHT \_\_\_\_\_ WEIGHT \_\_\_\_\_

ADDRESS \_\_\_\_\_

TELEPHONE \_\_\_\_\_ EMAIL \_\_\_\_\_

1. How often do you exercise? \_\_\_\_\_ times/week

2. Describe the intensity of your exercise (circle one)

1 = none

2 = light (e.g. casual walking, golf)

3 = moderate (e.g. brisk walking, jogging, cycling, swimming)

4 = heavy (e.g. running, high intensity sport activity)

3. What types of exercise do you engage in and how much do you do each session?

1 = none

2 = walking \_\_\_\_\_ km or minutes

3 = jogging/running \_\_\_\_\_ km or minutes

4 = swimming \_\_\_\_\_ meters or minutes

5 = cycling \_\_\_\_\_ km or minutes

6 = team sports (rugby, cricket, soccer, etc.) \_\_\_\_\_ minutes \_\_\_\_\_ intensity

7 = racquet sports \_\_\_\_\_ minutes

8 = weight training \_\_\_\_\_ minutes \_\_\_\_\_ # reps \_\_\_\_\_ # sets

9 = other \_\_\_\_\_

4. How much time per week do you spend exercising? \_\_\_\_\_ hours/week

5. Do you measure your heart rate during exercise? \_\_\_\_\_

If yes:

a. How high does it get during your typical workout? \_\_\_\_\_ beats/min

b. What heart rate is maintained throughout most of your workout?  
\_\_\_\_\_ beats/min

6. How long have you had a regular exercise program? \_\_\_\_\_

7. What condition or shape do you consider yourself to be in now (in terms of physical fitness)?

1 = poor

2 = fair

3 = good

4 = excellent

8. Do you or have you ever smoked? \_\_\_\_\_

If yes: How long ago? \_\_\_\_\_ For how many years? \_\_\_\_\_ How many packs/day?



9. How much and what type of alcohol do you consume in an average week?

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10. Has a close blood relative had or died from heart disease or related disorders (Heart Attack, Stroke, High Blood Pressure, Diabetes etc.)?

1=Mother

2=Father

3=Brother - Sister

4=Aunt - Uncle

5=Grandmother - Grandfather

6=None

If yes- Give ages at which they died or had the event and the problem they had.

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11. Have you ever had your cholesterol measured?

1=yes

2=no

If yes- write the date and value (or if it was normal or abnormal)

---

12. Indicate which of the following apply to you (circle all that apply).

1 = high blood pressure

2 = high blood fats or cholesterol

3 = cigarette smoking

4 = known heart disease or abnormalities

5 = family history of heart disease (parents or siblings before age 50)

6 = sedentary lifestyle

7 = stressful lifestyle at home or at work

8 = diabetes mellitus

9 = gout (high uric acid)

10 = obesity

13. Any medical complaints now (illness, injury, limitations)?

1 = yes      If yes, describe completely \_\_\_\_\_

2 = no      \_\_\_\_\_

\_\_\_\_\_

14. Any major illness in the past?

1 = yes      If yes, describe completely \_\_\_\_\_

2 = no      \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

15. Any surgery or hospitalization in the past?

1 = yes      If yes, describe completely \_\_\_\_\_

2 = no      \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

16. Are you currently taking any medications (prescription or over-the-counter: including birth control)?

1 = yes      If yes, list drugs and dosages \_\_\_\_\_

2 = no      \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_

17. Are you allergic to any medications?

1 = yes      If yes, list medications \_\_\_\_\_

2 = no      \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_

18. Have you ever had any neurological problems?

1 = yes      If yes, describe completely \_\_\_\_\_

2 = no      \_\_\_\_\_

\_\_\_\_\_

19. Do you now have, or have you ever had, any of the following? (circle all that apply)

1 = heart murmurs

2 = any chest pain at rest

3 = any chest pain upon exertion

4 = pain in left arm, jaw, neck

5 = any palpitations

6 = fainting or dizziness

7 = daily coughing

8 = difficulty breathing at rest or during exercise

9 = any known respiratory diseases

Please describe fully any items you circled\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

20. Do you now have, or have you ever had, any of the following? (circle all that apply)

1 = any bone or joint injuries

2 = any muscular injuries

3 = muscle or joint pain following exercise

4 = limited flexibility

5 = any musculoskeletal problems which might limit your ability to exercise

Please describe fully any items you circled\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_